

TOBACCO PHOSPHOLIPASE D β 1:
MOLECULAR CLONING AND BIOCHEMICAL CHARACTERIZATION

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Transgenic tobacco plants were developed containing a partial PLD clone in antisense orientation. The PLD isoform targeted by the insertion was identified. A PLD clone was isolated from a cDNA library using the partial PLD as a probe: Nt10B1 shares 92% identity with PLD β 1 from tomato but lacks the C2 domain.

PCR analysis confirmed insertion of the antisense fragment into the plants: three introns distinguished the endogenous gene from the transgene. PLD activity was assayed in leaf homogenates in PLD β / γ conditions. When phosphatidylcholine was utilized as a substrate, no significant difference in transphosphatidylolation activity was observed. However, there was a reduction in NAPE hydrolysis in extracts of two transgenic plants. In one of these, a reduction in elicitor-induced PAL expression was also observed.

TABLE OF CONTENTS

	Page
LIST OF TABLES.....	iv
LIST OF ILLUSTRATIONS.....	v
ABBREVIATIONS.....	vi
Chapter	
1. INTRODUCTION.....	1
Molecular Analysis of PLD	
PLD Protein Domains	
Downstream Regulation	
Evidence for PLD Activity in Fungal Elicitor Perception	
Research Rationale and Objectives	
2. MATERIALS AND METHODS.....	9
Screening the Tobacco Cell Line cDNA Library	
DNA Sequencing and Analysis	
Subcloning Nt10B1	
Colony Screening and Plasmid Extraction	
Protein Expression	
Protein Isolation and SDS PAGE	
Seed Germination	
Transformation Vector	
Tobacco Transformation	
DNA Isolation	
Polymerase Chain Reaction (PCR)	
Subcloning the Genomic Region PCR Product	
Leaf Homogenization	
Estimation of Protein Content	
Chemicals for PLD Activity Assays	
PLD Activity Assays	
Transphosphatidylation	
Hydrolysis of NAPE	
Lipid Extraction	

Thin Layer Chromatography (TLC)
Seedling Root Growth

3. RESULTS.....	26
The cDNA Clone Nt10B1: a Putative PLD β 1	
Subcloning of Nt10B1	
Expression and Transphosphatidylation Activity of the Clones	
Protein Isolation and SDS PAGE	
Identification of Transgenic Tobacco Plants	
Segregation Study of the T ₁ Progeny	
PLD Activity Screen of the Antisense PLD Plants	
Root Growth Rate	
4. DISCUSSION.....	67
APPENDIX.....	76
REFERENCES.....	78

LIST OF TABLES

Table	Page
1. Numbers of germinated ASPLD 7, 9, 11 and 13 T1 seedlings on kanamycin.....	58
2. <i>Arabidopsis thaliana</i> PLD β 1 similarity at the DNA level with other <i>Arabidopsis</i> PLD isoforms over the region that shares identity with the partial tobacco PLD clones (AF195614) used to generate the antisense (ASPLD) plants.....	69

LIST OF ILLUSTRATIONS

Figure	Page
1. The pB121 vector.....	16
2. pBI121-ASPLD β 1: the PLD antisense construct used to generate transgenic tobacco plants.....	17
3. The DNA sequence of the putative PLD clone Nt10B1 (Accession No. AY138861) and the corresponding predicted amino acid sequence.....	29
4. Alignment of the predicted amino acid sequence of Nt10B1 with other plant PLDs.....	35
5. Conserved <i>Arabidopsis</i> PLD protein domains in general and in Nt10B1....	38
6. Detailed conserved PLD protein motifs in <i>Arabidopsis</i> and Nt10B1.....	40
7. Screening post-transformation colonies by PCR to identify colonies with an insert (Expres1.2) of the correct size.....	42
8. Screening post-transformation colonies by PCR to identify colonies with an insert (Expres1.8) of the correct size.....	44
9. Expression of subcloned Nt10B1 products (Expres1.2 and Expres1.8) in <i>E.coli</i> and transphosphatidylation results.....	49
10. Confirming the incorporation of the transgene in the plant genome by PCR analysis.....	53
11. Transgenic ASPLD7, 9, 11, and 13 seeds (T1 generation) germinated to investigate segregation of the transgene.....	57
12. Transphosphatidylation of ethanol and NAPE hydrolysis: both assays were used to measure PLD activity in the transgenic plants.....	61
13. Root growth rate of wt and ASPLD tobacco T ₁ seedlings.....	66

ABBREVIATIONS

PLD – Phospholipase D

ASPLD – Antisense Phospholipase D

NAPE - *N*-Acylphosphatidylethanolamine

NAE - *N*-Acylethanolamine

PAL2 – Phenylalanine-ammonia lyase

PIP2 - Phosphatidylinositol-bisphosphate

PPIs - Polyphosphoinositides

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

CHAPTER 1

INTRODUCTION

Phospholipase D (PLD) is a ubiquitous enzyme in bacteria, plants, animals and yeast (Munnik *et al.*, 1998). Although much is known about its catalytic regulation, a precise physiological role for PLD in plants remains unclear. It was until recently regarded as an enzyme that degraded membrane phospholipids (Wang, 1997). However, research has revealed that it is involved in a number of signaling cascades such as those involved in seed germination (Wang, 1993; Ryu *et al.*, 1996; Ritchie and Gilroy, 1998), senescence (Fan *et al.*, 1997; Thompson *et al.*, 1998), response to water stress (Maarouf *et al.*, 1999; Frank *et al.*, 2000) specifically in the regulation of stomatal guard cells (Sang *et al.*, 2001), chilling (Pinhero *et al.*, 1998), wounding (Ryu *et al.*, 1997) and pathogen attack (Young *et al.*, 1996; Chapman *et al.*, 1998). Recent molecular and biochemical studies have yielded much information as to its primary structure (Wang, 2000; Chapman *et al.*, 1998), its substrate specificity and although models are much more developed for its function in animal systems, the general picture of how it may function in cell signaling in plants is unfolding. Furthermore, current evidence indicates that a phospholipase D from tobacco binds to microtubules and the plasma membrane thus implicating a potential role for the enzyme bound to the cytoskeletal elements of the cell (Gardiner *et al.*, 2001). There are other reports of PLD associated with the plasma membrane. It has been shown that the enzyme relocates to the plasma membrane in rice

upon attack by a bacterial pathogen, specifically to the region surrounding the point of inoculation (Young *et al.*, 1996). It has therefore been hypothesized that PLD may in fact be involved in cytoskeletal-mediated vesicle trafficking to the plasma membrane (Munnik and Musgrave, 2001). PLD hydrolyzes the terminal bond of phospholipids to yield phosphatidic acid (PA) and a water-soluble head-group. PLD can also catalyze a transphosphatidylation reaction in which primary alcohols are supplied as a substrate and the end product is a phosphatidylalcohol rather than PA. PLD in fact forms a covalent bond with the PA intermediate during transphosphatidylation, releasing only the head group at first. It has been hypothesized that the phosphatidylated form of the enzyme could serve to anchor the complex in the membrane (Munnik and Musgrave, 2001). Furthermore, phosphatidylinositol-bisphosphate (PIP₂) is required for PLD activity of both the beta and gamma isoforms. PIP₂ can also serve as a membrane attachment site for proteins involved in membrane trafficking (Sang *et al.*, 2001). The involvement of plant PLD in membrane trafficking is just a hypothesis at present. However, it might hold the key to a major physiological role for PLD in plants.

Molecular Analysis of PLD

PLD genes have been cloned from castor bean (Accession No. Q41142), rice (Accession No. D73411 and AB001920), maize (Accession No. D73410), *Arabidopsis* (Accession No. U36381, U84568, AF138281, AF027408, AB031047, AF322228 and AF411833), cabbage (Accession No. AF090444 and AF090445), tobacco (Accession No. AF195614), tomato (Accession No. AF201661, AY013252, AY013253, AY013254, AY013255 and AY013256), cotton (Accession No. AF159139), cowpea (Accession No.

U92656), black-eyed pea (Accession No. U92656), *Pimpinella brachycarpa* (Accession No. U96438), and resurrection plant (Accession No. AJ133000 and AJ133001) (Wang, 2000; Frank *et al.*, 2000). The current classification of plant PLDs was established for the *Arabidopsis* PLD family based on both the catalytic properties and the primary sequence of the different PLD isoforms. *Arabidopsis* PLD α , β , γ , δ and ζ are placed in distinct groups (Wang, 1997; Wang, 2000; Wang and Wang, 2001; Qin and Wang, 2002). This classification now extends to all known plant PLDs and is based on sequence similarity. The differences in nucleotide sequence are used to class the PLDs cloned from rice (Accession No. D73411 and AB001920), maize (Accession No. D73410) and castor bean (Accession No. Q41142) to the PLD α group. Two members of the PLD β group, PLD β 1 and β 2, were recently cloned from tomato (Accession No. AY013255 and AY013256) (Laxalt *et al.*, 2001). In contrast, only two distinct isoforms of mammalian PLDs (PLD1 and PLD2) are known and they have been cloned from mice (Accession No. AY081194, NM_008156, and AF050666), rats (Accession No. NM_033299, D85729, D85728 and D85727) and humans (Accession No. NM_001503, AF033850, L11701 and L11702) (Wang, 2000).

PLD Protein Domains

More recently, attention has been focused on the conserved domains found amongst plant PLDs. All plant PLDs are known to contain two “HKD” motifs. Although these are approximately 300 amino acids apart, once the protein has folded they are thought to interact to form the catalytic site of the enzyme (Qin and Wang, 2002).

Other domains have been identified such as the “*TYIENQFF*” motif. This motif is said the most conserved domain among the 12 *Arabidopsis* PLDs (Qin and Wang, 2002). It has been suggested that this region is a potential site for the binding of phosphatidylcholine (PC) through interactions with the methyl group of the choline headgroup (Qin and Wang, 2002). PLDs that belong to either the β or γ isoforms contain a stretch of basic and hydrophobic residues that have been postulated as a potential PIP₂-binding site (Qin and Wang, 2002). Finally, most PLDs have a C2 calcium-binding domain near the amino terminus of the protein. C2 domains of other proteins are also known to bind phospholipids, polyphosphoinositides (PPIs) and proteins (Zheng *et al.*, 2000). The C2 domain varies somewhat between isoforms and has been suggested to determine the calcium range in which the protein is optimally active (Qin and Wang, 2002). PLD β , γ and δ have the most number of calcium-binding acidic residues within the C2 region (although PLD δ activity is independent of calcium) whereas PLD α has less and PLD α 4 has none at all.

PLD in Signal Transduction Pathways - Upstream Regulation

Research in animal systems has shown that PLD is an integral part of signal transduction pathways. Likewise it is suspected that PLD is also involved in signal transduction in plants. The requirement for Ca²⁺ and PPIs suggest an upstream regulation of PLD activity. PLD1 activation by small G-proteins such as members of the ARF and Rho families is well-documented in animal cells (Hammond *et al.*, 1995; Hammond *et al.*, 1997), and evidence indicates that G-proteins activate PLD in plants including a

direct interaction of PLD and G-proteins in tobacco (Munnik *et al.*, 1998; Ritchie and Gilroy, 2000; Lein and Saalbach, 2001).

Downstream Regulation

There is evidence that the phosphatidic acid released by PLD activity is involved in signal transduction (Munnik *et al.*, 1998). PA has been shown to be a potent cellular mediator (Munnik, 2001) and phosphatidate phosphohydrolase can convert it to diacylglycerol (DAG) (Wang *et al.*, 1993). DAG is an activator of protein kinase C (PKC). The PA generated by PLD hydrolysis could therefore indirectly mediate many cellular events via PKC. PA can also be hydrolyzed by some members of the phospholipase A family leading to the generation of lysophosphatidic acid (LPA) and a free fatty acid. LPA is a proposed secondary messenger in mammalian systems and free fatty acids such as linoleic and linolenic acids can be used in the octadecanoid pathway. The octadecanoid pathway leads to the production of the jasmonic acid, which is a volatile lipid involved in plant stress and defense signaling. This pathway has also been suggested as a possible downstream cascade initiated by PLD (Young *et al.*, 1996).

Since PLD has been shown to be associated with the cytoskeletal elements of the plant cell (Young *et al.*, 1996; Wang and Wang, 2001), including more specifically the microtubule portion and the plasma membrane (Gardiner, 2001), it may be that this enzyme is also involved in trafficking important elements of the stress/wound response to the plasma membrane. In this manner, PLD could also be involved downstream of a wounding event, either to bring elements needed for cell/membrane repair to the site of

attack or as part of the signal transduction pathway coordinating the assembly of further signaling elements at the cell membrane.

Evidence for PLD Activity in Fungal Elicitor Perception

Recent reports have shown that upon treatment with a fungal elicitor, xylanase, there is a rapid release of N-acyl ethanolamines (NAEs) and a compensatory decrease in cellular N-acylphosphatidylethanolamine (NAPE) in both cultured tobacco cells (Chapman, 1998) and tobacco plants (Tripathy *et al.*, 1999). Furthermore, NAEs were shown to induce expression of defense gene encoding phenylalanine-ammonia lyase (PAL2) a key regulatory enzyme in the phenylpropanoid pathway involved in the synthesis of defense-related phytoalexins (Tripathy *et al.*, 1999).

In the presence of PIP₂ and phosphatidylethanolamine (PE), recombinant *Arabidopsis* PLD β and γ were able to hydrolyze PC, phosphatidylglycerol (PG), and NAPE (Pappan *et al.*, 1998). PE has been suggested to alter substrate presentation to allow the reaction with PC, PG and NAPE. PLD β shows a preference for PC over NAPE whereas PLD γ displays a preference for NAPE over PC (Pappan *et al.*, 1998). It is therefore possible to hypothesize that PLD γ (and possibly PLD β) are involved in catalyzing the conversion of NAPE to NAE in the response to fungal elicitors observed in tobacco and thus indirectly induce the expression of the plants defense genes. To further investigate this possibility, transgenic tobacco plants were developed containing a PLD fragment inserted in the antisense orientation with respect to endogenous gene. The 1.2 kb fragment has amino acid sequence identity with *Arabidopsis* PLD isoforms β (72%) and γ (68%). Using the transgenic plants as tools, I extended the work of others in the

lab to characterize transgenic tobacco plants and to assess the impact of antisense PLD constructs on endogenous PLD activity.

Research Rationale and Objectives

In order to investigate the role of PLD in plants, transgenic tobacco plants were developed by Dr. Swati Tripathy containing a partial PLD clone (Accession no. AF195614) in the antisense orientation. I aimed to identify the PLD isoform encoded by the DNA fragment (Accession no. AF195614) to determine which PLD isoform was most likely affected by the insertion of the antisense fragment. Toward this end additional PLD cDNAs were isolated from a tobacco cell cDNA library using the smaller fragment as a probe. The cDNA library screen yielded a longer partial PLD clone. Upon sequencing, this clone was found to be identical to the smaller fragment used to screen the library with the addition of 163 predicted amino acids at the *C* terminus containing the second “*HKD*” catalytic motif. Overall the new clone Nt10B1 (Accession No. AY138861) shares 92% identity at the amino acid level with PLD β 1 from tomato but lacked the C2 calcium-binding domain at the *N* terminus. Furthermore, the region of interest of Nt10B1 was subcloned into *E.coli* to test for functional PLD transphosphatidylase activity in PLD β conditions.

My second goal was to identify T₀ transgenic plants carrying the antisense PLD β construct. This was achieved using a PCR strategy that enabled us to distinguish the endogenous gene(s) from the transgenic insert based on the presence of introns in the region of the endogenous gene when PCR amplified. The PCR product from the endogenous gene was excised, cloned and sequenced for further confirmation. The region

amplified by PCR was found to contain three introns. Furthermore, T₁ seed was germinated on kanamycin to study the segregation of the transgene within the transgenic population.

Thirdly, our goal was to study *in vitro* PLD β/γ activity in extracts from these transgenic T₀ tobacco plants as compared to wild type tobacco extracts. PLD β/γ activity measured as transphosphatidylation was not significantly different in the transgenic plants versus the wild types. However, when measured as hydrolysis of NAPE, PLD β/γ activity was significantly reduced in two of the transgenic plants. Although the transgenic plants did not overtly show any morphological differences when compared with wild type tobacco plants, physiological effects of the transgene were noticed in our preliminary investigations of seedling root growth rate and PAL expression in the mature plants.

CHAPTER 2

MATERIALS AND METHODS

Screening the Tobacco Cell Line cDNA Library

The tobacco cell line cDNA library was kindly provided by Dr. Gyn An (Washington State University). The library was constructed from mRNA extracted from an early exponential growth stage NT1 tobacco cell line. The average insert size was 1 kb and *EcoRI* and *NotI* linkers (purchased from Pharmacia, NY) were ligated at either end of each insert. λ ZapII was used as the vector provided with the Uni-ZAP[®] XR Library (Stratagene, CA). The cDNA library was screened using Colony/Plaque Screen[™] Hybridization Transfer Membranes (NEN Life Sciences, MA). The primary screen (>30,000 plaques/plate) was performed by Heath Wessler using the partial PLD clone AF195614 radiolabelled with ³²P. I performed the secondary, tertiary and quaternary screens using the partial PLD clone AF195614 labeled and detected with the DIG High Prime DNA labeling and Detection and Detection Kit II (Roche, IN), which randomly primes DNA labeling with a digoxigenin-dUTP (alkali-labile). Hybridization was detected via an enzyme immunoassay with CSPD[®], a chemiluminescent substrate for alkaline phosphatase. The kit was used as per the manufacturers instructions. Chemiluminescence was detected by exposing the hybridized membranes to Kodak Scientific Imaging X-Omat Film[™] AR (Kodak, NY). The clones of interest were excised in vivo from the UniZAP XR vector. XL1-Blue MRF' *E. coli* cells were co-infected with

the ExAssist helper phage and the library phage containing the cDNA of interest. The ExAssist helper phage is designed to allow efficient excision of the phagemid pBluescript with the cDNA fragment of interest from the Uni-ZAP XR vector. SOLR *E. coli* cells are then transformed with the excised phagemids. The SOLR cell line is altered so as to prevent replication of the helper phage. pBluescript phagemid was then extracted using the Wizard[®] Plus SV Miniprep DNA Purification System (Promega, WI). Phagemids were subsequently cloned into chemically competent TOP10 cells (50 mL mid Log Phase cell pellet washed in 25 mL 0.1 M calcium chloride, 0.01 M rubidium chloride (CaRb) for 30 min on ice then resuspended in 2 mL CaRb and used in 200 µl aliquots). Phagemid was extracted for sequencing using the Quiagen Plasmid Midi Kit (Quiagen, CA) as per the manufacturers protocol with the exception that DNA was precipitated with isopropanol overnight.

DNA Sequencing and Analysis

The cDNA inserts of interest within the excised pBluescript phagemids were sequenced using the IRD-41 labeled M13 Forward and Reverse primers (LI-COR, NE), using a Sequi-Therm EXCEL II Kit-LC fluorescent-labeled primer (Epicentre Technologies, WI) and processed on an automated LI-COR sequencer (Li-COR 4000). Additional sequencing was carried out by Lone Star Laboratories, Inc. (Houston, TX). All DNA sequences were confirmed by completely sequencing both DNA strands. Analysis of nucleotide and amino acid sequences were performed using DNASIS software (HIBIO DNASIS for Windows, version 2) and BLAST programs (www.ncbi.nlm.nih.gov).

Subcloning Nt10B1

The region of interest was amplified by PCR using the Taq polymerase (Promega, WI) and Nt10B1 in pBluescript as template. The primer ExpresF2 (5'-GCTCGTCGGTTGATTACATTAC-3') was designed to bind at the 5' end of the sequence of interest. PLDR and the universal T7 primer were used respectively as primers for the 1.2 kb and 1.8 kb products. PCR conditions were as follows for the Perkin Elmer 2400 Thermal Cycler (Perkin Elmer, CT). The DNA was initially denatured at 95°C for 45 sec. The regions of interest were amplified using 32 cycles of 95°C for 45 sec to denature the DNA, 42°C for 45 sec to enable primers to bind to the template and 72°C for 2 min to enable elongation by the polymerase. A further 10 min at 72°C were allowed to ensure full-length products. The 1.2 kb and 1.8 kb PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The corresponding band was excised from the gel and purified using the Prep-A-Gene[®] DNA Purification System (Bio-Rad, CA) for DNA isolation from agarose gel slices. The resulting DNA was ligated into the vector pTrcHis or pTrcHis2 (Invitrogen, CA) using the pTrcHis[®] TOPO[®] PCR Cloning Reaction (Invitrogen, CA) and the resulting vector was introduced into chemically competent One Shot[™] *E. coli* TOP10 cells (Invitrogen, CA). The transformation mixture was plated overnight on LB plates with 50 µg/mL ampicillin and 0.5% glucose to repress gene expression.

Colony Screening and Plasmid Extraction

Ampicillin-resistant colonies were selected. The colonies were touched with a sterile loop, which was briefly dipped in 50 µl sterile ultrapure water for colony PCR

screening. The same loop was then used to streak the colony (first streak only) on a fresh LB plate (containing 50 µg/mL ampicillin and 0.5% glucose) and to inoculate liquid cultures. Liquid cultures were grown in LB broth with 50 µg/mL ampicillin. Plates were incubated at 37°C overnight then stored at 4°C. Ten colonies were routinely selected in this manner per transformation. The 50 µl sample containing the bacterial cells in water was boiled for 5 min to lyse cells. The sample was then used as template for a PCR reaction to determine whether the colony contained the vector with an insert of the correct size. The PCR reaction primers and conditions were identical to those used to generate the insert for ligation into pTrcHis and pTrcHis2 with the exception of the template. In the case of the 1.8 kb fragment in pTrcHis, the pTrcHis Xpress™ Forward primer was used along with the ExpresF2 primer to determine if the insert had been inserted in the correct orientation. Plasmid DNA was isolated from colonies that tested positive using the Wizard® Plus MiniprepDNA Purification System (Promega, WI). Plasmid DNA was sequenced by Lone Star Laboratories, Inc.(Houston, TX).

Protein Expression

Liquid cultures (25 mL) were grown at 37°C with shaking at 280 rpm (Innova 4000 Incubator Shaker, New Brunswick Scientific) to an OD of 0.6 at 600 nm (around 3h). IPTG was added to a final concentration of 1 mM and cells were grown overnight at 37°C with shaking at 280 rpm. Cells were pelleted at 7,000xg for 10min. The supernatant was discarded and cells were resuspended in 3 mL 50 mM Tris-HCl (pH 8.0, 150 mM NaCl, 0.25 mM PMSF, 2 mM EDTA. Cells were lysed by sonicating on ice (three 10 sec bursts at setting 4). The lysate was centrifuged at 10,000xg at 4°C for 5 min to remove

cell debris. The supernatant was used for protein quantification, PLD activity assays, and/or protein isolation.

Protein Isolation and SDS PAGE

Proteins of interest were isolated using the denatured protein protocol of the ProBond™ Purification System (Invitrogen, CA) as per the manufacturer's instructions. Isolated protein was pooled and washed/concentrated using the Centricon® YM-30 Centrifugal Filter Devices (Millipore, MA). Proteins were then quantified using the Bradford assay (Bradford, 1976), denatured in SDS and separated in a 10% Tris-HCl polyacrylamide gel electrophoresed at 35 mA for 15 min then 65 mA for approximately 30 min. To visualize bands, gels were stained in Coomassie blue (0.25% coomassie brilliant blue (w/v), 45.4% methanol (v/v), 4.2% acetic acid (v/v)) overnight.

Seed Germination

Tobacco seeds were sterilized in 10% bleach for 5 min and rinsed twice in sterile water. Using sterile toothpicks, the seeds were placed at regular intervals into growth media plates 10% MS Micronutrient Solution (v/v) (Murashige and Skoog, 1962), 10% MS Macronutrient Solution (v/v) (Murashige and Skoog, 1962), 3% sucrose (w/v), 112 mg/L vitamin B5, pH 5.8 and 2.3 g/L phytoGel). Transgenic seeds were selected by germination and growth in 100 µg/mL kanamycin. The seeds were placed under growth lights (30 W Philips–Alto Collection) in 16 h photoperiod at 25-30°C for at least 20 days and germination numbers were recorded. For some experiments kanamycin was not included in the media to score seed germination rates and record seedling growth measurements.

Binary Vector for Tobacco Transformation

The vector, designated ASPLD, was constructed in pBI121 (Figure 1) by Heath Wessler in Dr. Benjamin's Laboratory for use in tobacco transformation. The ASPLD vector (Figure 2) was constructed with a PCR product generated using primers 5'-GGGAAGTGCTGGGAGGAC-3' and 5'-TTTTTTCATAAGAGACTCATCGTC-3' (shown in red on figure 3) designed specifically to the partial PLD cDNA clone AF195614. The PCR product was inserted in reverse orientation into pBI121. pBI121 contains the Ti-plasmid left and right border (LB, RB) inverted repeat sequences that enable the integration of the DNA into the tobacco genome; the neomycin phosphotransferase II (NPTII) gene that confers resistance to kanamycin under regulation of the nopaline synthetase (NOS) promoter and the β -glucoronidase (GUS) gene under the regulation of the cauliflower mosaic virus (CaMV) 35S promoter. pBI121 was digested with the restriction enzymes *SacI* and *BamHI* to remove the GUS encoding region and the PCR-generated fragment in reverse orientation was cloned in its place. The binary vectors were introduced by Heath Wessler into *Agrobacterium tumefaciens* (strain LBA4404) and maintained with kanamycin selection.

Tobacco Transformation

Plant transformation was performed by Dr. Swati Tripathy following the procedure by Svab *et al.*, 1995, with some modifications. Transgenic plants were regenerated from independent calli lines via organogenesis and were transferred to soil for maturation and seed production in a growth room under 14 h photoperiod at 25-30°C.

DNA Isolation

DNA from young leaves of transgenic and control plants was extracted according to Patterson *et al.* (1993). Leaf material was collected and immediately placed on ice. The tissue was washed and weighed once all significant veins had been cut out. Around 4g of leaf tissue was used per sample. The tissue was homogenized in a minichop blender on ice with DNA extraction buffer (20 mL ice-cold 0.35 M glucose, 0.1 M Tris-HCl (pH 8.0), 0.005 M Na₂EDTA (pH 8.0), 2% polyvinylpyrrolidone (PVP) (w/v), 0.1% diethyldithiocarbamic acid (DIECA) (w/v) with 0.1% ascorbic acid (w/v) and 0.2% mercaptoethanol (w/v) added immediately before use). The blender was pulsed twice for 10s. Samples were centrifuged at 2700xg for 20 min at 4°C (SS-34 rotor, Sorvall RC 5C). The nuclei-containing pellet was recovered and the nuclei were lysed in 8 mL nuclei lysis buffer (0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 0.02 M Na₂EDTA (pH 8.0), 2% hexadecyltrimethylammonium bromide (CTAB), 2%PVP (w/v), 0.1 %DIECA (w/v) with 0.1% ascorbic acid (w/v) and 0.2% mercaptoethanol (w/v) added immediately before use) at 65°C for 30 min. Chloroform-isoamyl alcohol (CIA) (10mL, 24:1) was added to each sample to remove proteins and the resulting solution was then inverted 50 times and centrifuged at 2700xg to accelerate the phase separation. The aqueous phase was removed and washed 2 more times with CIA. The final aqueous phase was transferred to a clean tube and the DNA was precipitated with 0.6 volumes isopropanol. The DNA was pelleted at 10,000xg and washed with 1 mL ethanol. The pellet was resuspended in 1 mL TE buffer (10mM Tris, 1mM EDTA, pH 8.0) at 55°C for 45 min with brief vortexing every 10 min. The purity of the DNA sample was estimated at 260 nm and 280 nm.

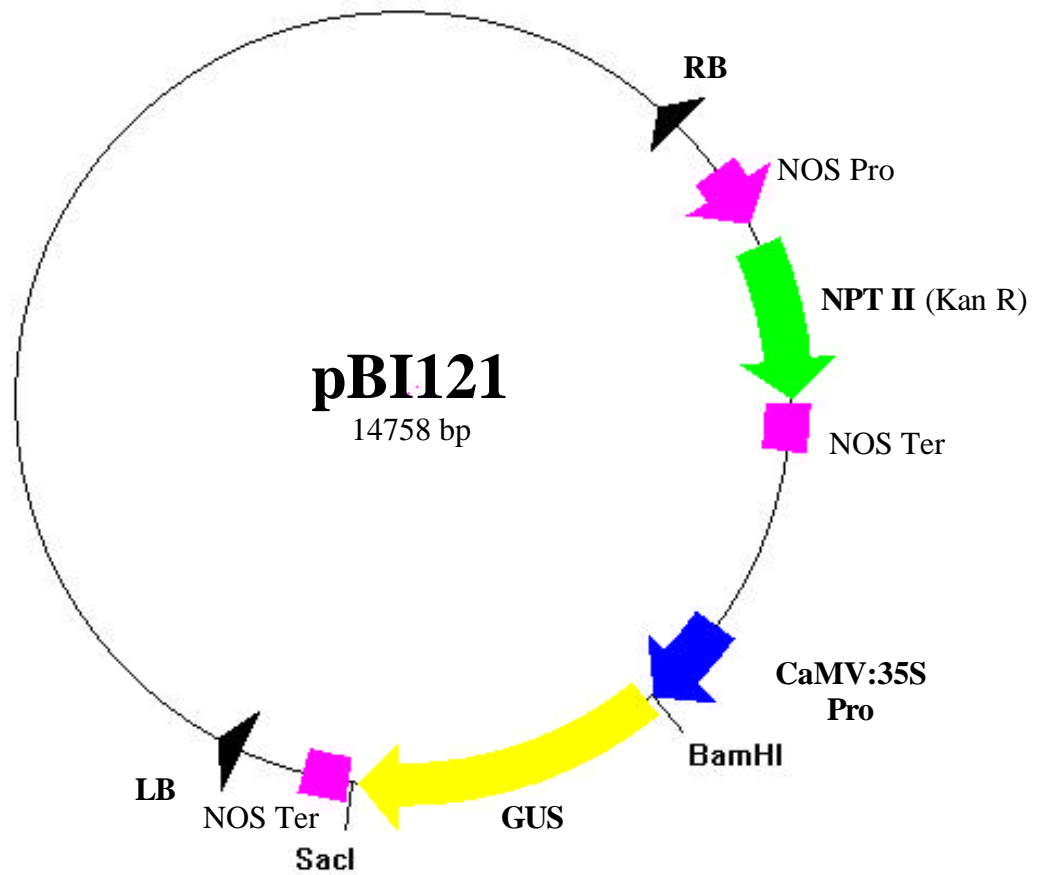


Figure 1. The pBI121 vector purchased from Clontech and redrawn from Cheng and Yeh, 2000. The neomycin phosphotransferase II (NPTII) gene under regulation of the nopaline synthetase (NOS) promoter conveys resistance to the antibiotic kanamycin. The glucoronidase (GUS) gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

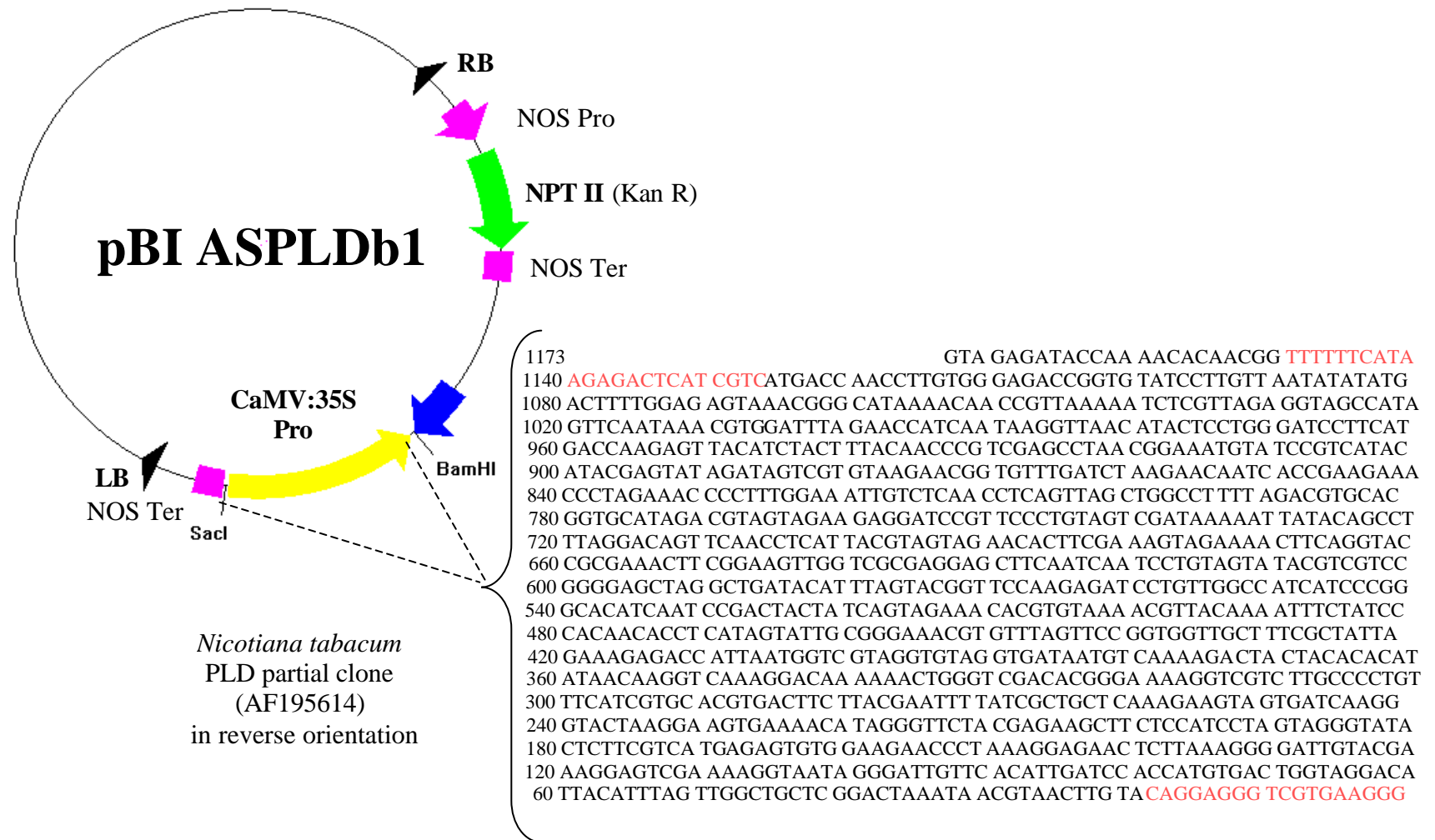


Figure 2. The PLD antisense construct used to generate transgenic tobacco plants (*T. xanthi*). The GUS gene was excised from pBI121 vector using the restriction enzymes *SacI* and *BamHI*, and the partial PLD clone (AF195614) was inserted into pBI121 in the antisense orientation behind the CaMV 35S promoter (Wessler and Benjamin unpublished). The red letters depict the primers used to PCR amplify the portion of the clone inserted into pBI121. The neomycin phosphotransferase II (NPTII) gene under regulation of the nopaline synthetase (NOS) promoter conveys resistance to the antibiotic kanamycin.

Polymerase Chain Reaction (PCR)

Nuclear genomic DNA samples were used as templates in PCR reactions with *Amplitaq[®]* DNA polymerase (Perkin Elmer) and primers specific for PLD. The following primers were designed to amplify the 601 base pair region of the partial PLD clone AF195614 (and found to amplify the 1086 base pair region of the endogenous gene): PLDF (5'-GCCTAACTACACGGGCCCTACTACCGGTTG-3') and PLDR (5'-GCAGTACTGGTTGGAACACCCTCTGGCCAC-3') (synthesized by Biosynthesis, Inc., TX). PCR conditions were as follows for Perkin Elmer 2400 Thermal Cycler. The DNA was initially denatured at 95°C for 45 sec. The region of interest was amplified using 32 cycles of 95°C for 45 sec to denature the DNA, 57°C for 45 sec to enable primers to bind to the template and 72°C for 2 min to enable elongation by the polymerase. A further 10 min at 72°C were allowed to ensure full-length products. The 601 bp and 1086 bp PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide.

Subcloning the Genomic Region PCR Product

The region of interest was PCR amplified using the DNA polymerase *Pfu Turbo* (Stratagene, CA) and nuclear genomic DNA extracted from wild type *Nicotiana tabacum* cv Xanthi as template. PLDF and PLDR were used as primers. PCR conditions were as follows. The DNA was initially denatured at 95°C for 5 min. The region of interest was amplified using 32 cycles of 95°C for 45 sec to denature the DNA, 57°C for 45 sec to enable primers to bind to the template and 72°C for 4 min to enable elongation by the polymerase. A further 20 min at 72°C were allowed to ensure full-length products. The

1086 bp PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The corresponding band was excised from the gel and purified using the Prep-A-Gene[®] DNA Purification System (Bio-Rad, CA) for DNA isolation from agarose gel slices. The resulting DNA was ligated into pZero using the Zero Blunt[®] TOPO[®] PCR Cloning Reaction (Invitrogen, CA) and the resulting vector was introduced into chemically competent One Shot[™] *E. coli* TOP10 cells (Invitrogen, CA). The transformation mixture was plated on LB plates with 50 µg/mL kanamycin overnight. Single colonies were selected and grown in LB broth with 50 µg/mL kanamycin. Plasmid was extracted using the Quiagen Plasmid Midi Kit (Quiagen, CA) as per the manufacturer's protocol with the exception that DNA was precipitated with isopropanol overnight. Plasmid DNA was sequenced by Lone Star Laboratories, Inc. (Houston, TX). Three clones were fully sequenced to verify sequence identity.

Homogenization of Leaves for Enzyme Assays

Young leaves were harvested and placed immediately on ice. The petiole and major veins were removed and leaf tissue was weighed. The tissue was then ground with a mortar and pestle on ice in homogenization buffer (10 mM KCl, 1mM EDTA, 1mM MgCl₂, 400 mM sucrose, 1 mM EGTA, 100 mM Kphos Buffer pH 7.2). The homogenate was filtered through two layers of cheesecloth. The final volume was recorded and samples were centrifuged for 20 min at 10,000xg at 4°C. The supernatant was recovered. The protein content of the sample was estimated and the crude homogenate was used in enzyme assays.

Estimation of Protein Content

Protein content was routinely estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the protein standard. Briefly, 10 μ l of the protein sample was mixed with 40 μ l water and 50 μ l of 1 N NaOH. To each sample, 1 mL Bradford reagent (Bradford, 1976) was added and vortexed thoroughly. Samples were incubated 15 min at room temperature. After vortexing again, the absorbance of the samples was measured at 595 nm in a spectrophotometer (Milton Roy Company, NY). Absorbances were used to calculate the protein content of the sample based on the BSA standard curve.

Chemicals for PLD Activity Assays

The 1,2-Dilauryl-*sn*-glycero-3-phospho(*N*-[2¹⁴-C]palmitoyl)ethanolamine (NAPE) was synthesized and kindly provided by Dr Gitte Pettersen. Phosphatidylcholine, L- α -dipalmitoyl, [dipalmitoyl-1-¹⁴C] was purchased from NEN Life Sciences, MA. L- α -phosphatidylethanolamine (dipalmitoyl, *N*-palmitoyl) (NAPE), L- α -phosphatidylcholine Type II from Soybean, L- α -phosphatidylethanolamine (dioleoyl), cabbage PLD Type V, *Streptomyces chromofuscus* PLD, bovine serum albumin, were from Sigma Chemical Co., MO. L- α -Phosphatidyl-D-myo-inositol-4,5-bisphosphate was obtained from either Roche Molecular Biochemicals, IN or Sigma Chemical Co., MO. All other reagents used in biochemical analysis were purchased from Fisher Scientific, PA, unless otherwise stated.

PLD Activity Assays

PLD activity was assayed as transphosphatidylation and NAPE hydrolysis in separate experiments. The conditions used were previously optimized for optimal transphosphatidylation activity and hydrolysis of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) *in vitro* (Pappan *et al.*, 1997 and Pappan *et al.*, 1998).

Transphosphatidylation by PLD (Figure 12A)

PLD transphosphatidylation activity was assayed by using phosphatidylcholine, L- α -dipalmitoyl, [dipalmitoyl-1- ^{14}C] (^{14}C -PC) and unlabeled phosphatidylcholine as a substrate. The assays took place in a total volume of 100 μl . This volume was composed of 25 μl of 4X assay buffer (final concentrations were 100 mM MES, 50 μM CaCl_2 , 2 mM MgCl_2 , 80 mM KCl, pH 7.0), and 25 μl of lipid master mix (3.6 μmol PE, 0.34 μmol PIP_2 , 0.28 μmol PC including 0.05 μCi ^{14}C -PC). The lipid master mix was mixed in advance, dried under nitrogen and resuspended in 25 μl ultrapure water with constant vortexing and sonicating to resuspend the lipid vesicles. 1.5 μl 95% ethanol was added to each reaction immediately before the reaction began. The reaction was started by the addition of 50 μg of protein from crude leaf homogenates in 50 μl volume (volumes were adjusted with ultrapure water). Samples were incubated at 30°C at 70 rpm in a shaking waterbath for 1 h unless otherwise indicated. The reaction was stopped with hot isopropanol (70°C) and lipids were extracted for analysis.

Hydrolysis of NAPE

PLD hydrolysis activity was assayed by using 1,2-dilauryl-sn-glycero-3-phospho(N-[2 14 C]palmitoyl)ethanolamine and unlabeled L- α -phosphatidylethanolamine (dipalmitoyl, *N*-palmitoyl) (NAPE) as a substrate. The assays took place in a total volume of 100 μ l. This volume was composed of 25 μ l of 4X assay buffer (final concentrations were 100 mM MES, 50 μ M CaCl_2 , 2 mM MgCl_2 , 80 mM KCl, pH 7.0), and 25 μ l of lipid master mix (3.6 μ mol PE, 0.34 μ mol PIP_2 , 0.28 μ mol NAPE including ^{14}C -NAPE). The lipid master mix was mixed in advance, dried under nitrogen and resuspended in 25 μ l ultrapure water with constant vortexing and sonicating to resuspend the lipid vesicles. The reaction was started by the addition of 50 μ g of protein from crude leaf homogenates in 50 μ l volume (volumes were adjusted with ultrapure water). Samples were incubated at 30°C at 70 rpm for 1 hour unless otherwise indicated. The reaction was stopped with hot isopropanol (70°C) and lipids were extracted for analysis. Activity was measured as hydrolysis of 1,2-dilauryl-sn-glycero-3-phospho(N-[2 14 C]palmitoyl)ethanolamine and was quantified as production of radiolabelled NAEs.

Lipid Extraction

Lipid extraction from assay reactions was based on previously described methods by Chapman and Moore (1993). Reactions were stopped by adding 2 mL hot isopropanol (70°C) and incubating at 70°C for 30 min. Samples were then cooled for 5 min. Chloroform (1 mL) and 700 μ l ultrapure water were added and samples were left to extract overnight at room temperature. Phases were partitioned by the addition of 1 mL of

chloroform and 2 mL of ultrapure water and centrifugation at 1200xg for 5 min. The aqueous phase was aspirated off and the chloroform-based layer was washed two more times with 2 mL of ultrapure water. The lipid-containing chloroform was then transferred to vials and dried under a steady flow of nitrogen. The lipids were later resuspended in 25 μ l of chloroform and analyzed by thin layer chromatography and radiometric scanning.

Thin Layer Chromatography (TLC)

One dimensional TLC was used to separate the lipid products of the enzyme assays. The lipid samples were applied (2x 5 μ l) to 20x20 cm silica gel G plates (Whatman, layer thickness 250 μ m). Phosphatidylcholine and phosphatidylethanol were separated by TLC in a chloroform:methanol:ammonium hydroxide (65:35:5) solvent system for approximately 55 min. NAPE and NAE separation was performed in a chloroform:methanol:ammonium hydroxide (80:20:2) system for around 40 min. The radiolabelled products were quantified as a percentage of total radioactive lipid by radiometric scanning (Bioscan 200 Imaging Scanner, Bioscan, DC). The radiospecific activity of the substrate was then used to calculate the enzyme activity and expressed as nmol/min/mg protein.

Seedling Root Growth

Tobacco seed were surface-sterilized in 10% bleach for 5 min. They were then rinsed twice in sterile water. Using sterile toothpicks, the seeds were planted at regular intervals into growth media plates (10% MS Micronutrient Solution (v/v) (Murashige and Skoog, 1962), 10% MS Macronutrient Solution (v/v) (Murashige and Skoog, 1962), 3% sucrose (w/v), 112 mg/L vitamin B5, pH 5.8 and 2.3 g/L phytogel). The seeds were

placed under growth lights (30W Philips–Alto Collection) in 16 h photoperiod at 25–30°C at a near-vertical angle to ensure roots would grow straight down and root length was recorded at 3, 6, 8 and 11 days after planting.

CHAPTER 3

RESULTS

The cDNA Clone Nt10B1: a Putative PLD β 1

The clone Nt10B1 (AY138861) was isolated by screening a tobacco cell line cDNA library with the partial PLD cDNA clone previously isolated in our lab (AF195614) as a probe. The first screen of the library yielded fifty-one positive plaques. Twelve of these were carried through to the fourth screen. Sequencing information revealed that of these, ten had identity with the partial PLD cDNA clone. However, eight of these were later determined to be only small fragments and a ninth clone that had identity at the 5' end was found to contain sequence of no identity to PLD further downstream and was later abandoned. The remaining clone, Nt10B1, was fully sequenced and was found to contain high sequence similarity with other known plant PLDs from base pair 601 through to a stop codon at base pair 2340 (Figure 3). Nt10B1 was found to share highest identity with PLD β 1 from tomato (92% at the amino acid level) and is highly homologous to other plant PLD β s and γ s (Figure 4). (This portion of the clone was later subcloned into the expression vectors pTrcHis and pTrcHis2 and amino acid numbers given in figures refer to the subclone.)

Plant PLDs are known to share conserved regions. The first to be recognized were the two catalytic motifs of the HxKxxxxD type. Nt10B1 contains both of these domains

(Figure 5). The first is located at amino acid 112 and the second more elaborate HxKxxxxDxxxxxGSANINQR at amino acid 467. Although they are nearly 300bp apart they are thought to interact with each other to form the catalytic site (Qin and Wang, 2002). Other domains have subsequently been identified (Qin and Wang, 2002). Of these Nt10B1 contains the “*YIENQFF*” motif (Figure 6). The aromatic amino acids suggest that this region is a potential site for the binding of PC through hydrophobic interactions with the methyl group of the choline headgroup (Qin and Wang, 2002). Finally, Nt10B1 contains the basic and hydrophobic residues that have been postulated as a potential PIP₂-binding site (Qin and Wang, 2002) (Figure 6). However, Nt10B1 lacks the 130 residue calcium/phospholipid-binding domain located at the *N* terminus of the protein in PLD β s, γ s, and δ s (Qin and Wang, 2002) (Figure 5).

Subcloning of Nt10B1

I postulated that a PLD without the calcium-binding domain may be active yet insensitive to calcium levels. To test this hypothesis and study the expression of Nt10B1, the region of interest (from 601 bp to 2640 bp) was PCR amplified and subcloned in frame into suitable expression vectors (and named Expres1.8). Furthermore, a second PCR product was amplified from Nt10B1 that would not encode the second catalytic site (from 601 bp to 1690 bp and named Expres1.2) (Figure 9A).

A primer was designed (expresF2) at the 5' end of the subsequence of Nt10B1 (the region homologous to other plant PLDs) for PCR amplification (Figure 4). The 3' primer used was either the universal T7 primer or the PLDR primer. The resulting products were respectively 1.8 kb in length and 1.2 kb length. Both fragments were ligated into the

expression vectors pTrcHis and pTrcHis2 and transformed into competent Top10 cells. Transformed colonies were screened by PCR using the same primers for inserts of the right size for the 1.2 kb fragment (Figure 7) and for the right size and orientation within the vector (using the pTrcHis Xpres™ Forward priming site) for the 1.8 kb fragment (Figure 12). Positive clones were confirmed by sequencing.

```

      10      19      28      37      46      55
5' TGC ATT ACT TCA TCA GTC AAG GAC AAA TAC AAA TCA GAA ACA CCA AAA ACT AAG
   ---
   C I T S S V K D K Y K S E T P K T K

      64      73      82      91      100      109
AAC CTT TAC ATT CAC CGA AAA TCC CAA AAT AAA AAC CTA AGA GCT AGT AAA CTT
   ---
   N L Y I H R K S Q N K N L R A S K L

      118      127      136      145      154      163
AGT AAC AGC CTT AGT CCC TTC AGA CAC AGC GTG CTT AGC CAA CTC CCC AGA CAA
   ---
   S N S L S P F R H S V L S Q L P R Q

      172      181      190      199      208      217
CAC GAG TCT CAC GGC AGT CTG AAT TTC CCT TGA AGT AAT TGT TGG CTT CTT ATT
   ---
   H E S H G S L N F P * S N C W L L I

      226      235      244      253      262      271
ATA CCT AGC TAA TCT AGA AGA TTC TTG AGC AAG CTT CTC AAA AAT ATC ATT AAT
   ---
   I P S * S R R F L S K L L K N I I N

      280      289      298      307      316      325
AAA ACT GTT CAT GAT CCC CAT TGC CTT ACT AGA AAT ACC AAT ATC TGG GTG CAC
   ---
   K T V H D P H C L T R N T N I W V H

      334      343      352      361      370      379
TTG CTT CAG CAC TTT GAA GAT GTA AAT CTT GTA GGT TTC AAC GCT CTT TTT TTG
   ---
   L L Q H F E D V N L V G F N A L F L

      388      397      406      415      424      433
CCC TCT TTT TCT TCT TGT CAG CTC CTG CTG CTC CGG CTT CCT TTG GGA GCT TCT
   ---
   P S F S S C Q L L L L R L P L G A S

      442      451      460      469      478      487
TGC CCG CTT TTA GTT TCT TCT CTG CTG GGG TTT TCT CTG CCT TTT TCT CCT CTG
   ---
   C P L L V S S L L G F S L P F S P L

      496      505      514      523      532      541
TTG CTA CTG GTT TTT TCT CAG CTG GCT TCT TCT CTG CCT TTG GTG CCA TTG TAT
   ---
   L L L V F S Q L A S S L P L V P L Y

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550	559	568	577	586	595
ATA GCT TTC ACA GAA AAA TGA ATA GAG GAT TGA GGC GGC CGC GAA TTC GCG GCC					
I A F T E K * I E D * G G R E F A A					
604	613	622	631	640	649
GCG GCT CGT CGG TTG ATT TAC ATT ACA GGA TGG TCA GTG TAC CAC CTA GTT ACA					
A A R R L I Y I T G W S V Y H L V T					
658	667	676	685	694	703
CTT GTT AGG GAT AAT GGA AAA GCT GAG GAA AGC ATG TTA GGG GAA ATT CTC AAG					
L V R D N G K A E E S M L G E I L K					
712	721	730	739	748	757
AGG AAA TCC CAA GAA GGT GTG AGA GTA CTG CTT CTC ATA TGG GAT GAT CCT ACC					
R K S Q E G V R V L L L I W D D P T					
766	775	784	793	802	811
TCT TCG AAG AGC ATC TTG GGA TAC AAA AGT GAA GGA ATC ATG GGA ACT AGT GAT					
S S K S I L G Y K S E G I M G T S D					
820	829	838	847	856	865
GAA GAA ACT CGT CGC TAT TTT AAG CAT TCT TCA GTG CAC GTG CTA CTT TGT CCC					
E E T R R Y F K H S S V H V L L C P					
874	883	892	901	910	919
CGT TCT GCT GGA AAA GGG CAC AGC TGG GTC AAA AAA CAG GAA ACT GGA ACA ATA					
R S A G K G H S W V K K Q E T G T I					
928	937	946	955	964	973
TAC ACA CAT CAT CAG AAA ACT GTA ATA GTG GAT GTG GAT GCT GGT AAT TAC CAG					
Y T H H Q K T V I V D V D A G N Y Q					
982	991	1000	1009	1018	1027
AGA AAG ATT ATC GCT TTC GTT GGT GGC CTT GAT TTG TGC AAA GGG CGT TAT GAT					
R K I I A F V G G L D L C K G R Y D					
1036	1045	1054	1063	1072	1081
ACT CCA CAA CAC CCT ATC TTT AAA ACA TTG CAA AAT GTG CAC AAA GAT GAC TAT					
T P Q H P I F K T L Q N V H K D D Y					
1090	1099	1108	1117	1126	1135
CAT CAG CCT AAC TAC ACG GGC CCT ACT ACC GGT TGT CCT AGA GAA CCT TGG CAT					

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H   Q   P   N   Y   T   G   P   T   T   G   C   P   R   E   P   W   H

      1144      1153      1162      1171      1180      1189
GAT TTA CAT AGT CGG ATC GAG GGG CCT GCT GCA TAT GAT GTC CTA ACT AAC TTC
-----
D   L   H   S   R   I   E   G   P   A   A   Y   D   V   L   T   N   F

      1198      1207      1216      1225      1234      1243
GAG GAG CGC TGG TTG AAG GCT TCA AAG CGC CAT GGA CTT CAA AAG ATG AAA GCT
-----
E   E   R   W   L   K   A   S   K   R   H   G   L   Q   K   M   K   A

      1252      1261      1270      1279      1288      1297
TCA CAA GAT GAT GCA TTA CTC CAA CTT GAC AGG ATT CCC GAC ATA TTA AAA ATA
-----
S   Q   D   D   A   L   L   Q   L   D   R   I   P   D   I   L   K   I

      1306      1315      1324      1333      1342      1351
GCT GAT GTC CCT TGC CTA GGA GAA GAT GAT GCA GAT ACG TGG CAC GTG CAG ATT
-----
A   D   V   P   C   L   G   E   D   D   A   D   T   W   H   V   Q   I

      1360      1369      1378      1387      1396      1405
TTC CGG TCG ATT GAC TCC AAC TCT GTT AAA GGT TTC CCC AAA GAT CCC AAA GAA
-----
F   R   S   I   D   S   N   S   V   K   G   F   P   K   D   P   K   E

      1414      1423      1432      1441      1450      1459
GCC ACT AAC AAG AAT CTA GTT TGT GGC AAG AAT GTG CTG ATA GAT ATG AGC ATA
-----
A   T   N   K   N   L   V   C   G   K   N   V   L   I   D   M   S   I

      1468      1477      1486      1495      1504      1513
CAT ACT GCC TAT GTA AAG GCA ATC CGA GCT GCC CAA CAT TTC ATC TAC ATT GAG
-----
H   T   A   Y   V   K   A   I   R   A   A   Q   H   F   I   Y   I   E

      1522      1531      1540      1549      1558      1567
AAC CAG TAC TTC CTA GGG TCC TCA TAC AAT TGG AAT AAC TAC CAA GAT TTA GGT
-----
N   Q   Y   F   L   G   S   S   Y   N   W   N   N   Y   Q   D   L   G

      1576      1585      1594      1603      1612      1621
GCA AAT AAC TTG ATA CCG ATG GAG ATT GCT CTA AAA ATT GCC AAC AAA ATA CGG
-----
A   N   N   L   I   P   M   E   I   A   L   K   I   A   N   K   I   R

      1630      1639      1648      1657      1666      1675
GCA AAT GAG AGG TTT TCA GTA TAT ATA ATT GTT CCT ATG TGG CCA GAG GGT GTT
-----
A   N   E   R   F   S   V   Y   I   I   V   P   M   W   P   E   G   V

      1684      1693      1702      1711      1720      1729

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... CCA ACC AGT ACT GCT ACT CAG AGA ATA CTT TTT TGG CAG CAC AAT ACC ATG CAG
-----
P T S T A T Q R I L F W Q H N T M Q

1738 1747 1756 1765 1774 1783
ATG ATG TAT GAG ACA ATT TAC AAA GCT TTA GTA GAG GTA GGG CTG GAA AAC ACA
-----
M M Y E T I Y K A L V E V G L E N T

1792 1801 1810 1819 1828 1837
TAT GAA CCC CAA GAC TAT TTG AAT TTC TTC TGC CTT GGC AAT CGC GAG GTT CAG
-----
Y E P Q D Y L N F F C L G N R E V Q

1846 1855 1864 1873 1882 1891
GAA GAT GGA AAC AAT ACA GTT GTC AAA AGT TCT AAG CCA ACT ACA CCT CAG GAA
-----
E D G N N T V V K S S K P T T P Q E

1900 1909 1918 1927 1936 1945
CTC AGT CAG AAA AGT CGA CGT TTT ATG ATA TAT GTA CAT TCA AAA GGA ATG ATA
-----
L S Q K S R R F M I Y V H S K G M I

1954 1963 1972 1981 1990 1999
GTG GAT GAT GAG TAT GTT ATA TTG GGA TCT GCA AAC ATT AAT CAA CGT TCT CTG
-----
V D D E Y V I L G S A N I N Q R S L

2008 2017 2026 2035 2044 2053
GAA GGC ACC AGA GAT ACT GAA ATT GCA ATG GGT TCA TAT CAG CCC CAT CAT ACA
-----
E G T R D T E I A M G S Y Q P H H T

2062 2071 2080 2089 2098 2107
TGG GCA ACC AAA CAC TCT CGA CCA CAT GGA CAG GTA TAC GGC TAT AGG ATG TCT
-----
W A T K H S R P H G Q V Y G Y R M S

2116 2125 2134 2143 2152 2161
CTG TGG GCA GAG CAC ACG GGA ACT CTT GAG CAA TGT TTT GAG CAT CCA GAG AGT
-----
L W A E H T G T L E Q C F E H P E S

2170 2179 2188 2197 2206 2215
CTT GAG TGT GTG AGA CGG ATA AGA GTA TTT GGC GAA CAC AAC TGG CTA CAA TAC
-----
L E C V R R I R V F G E H N W L Q Y

2224 2233 2242 2251 2260 2269
GCG GCT GAT GAA GTA ACT GAG ATG AGA GGC CAC CTT CTT AAG TAT CCT GTT GAA
-----
A A D E V T E M R G H L L K Y P V E

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2278	2287	2296	2305	2314	2323
GTT GAT CGA ACG GGA AAG GTC AAG TCA CTA CCT GGC TGT GAA ACT TCC CAG ATA					
---	---	---	---	---	---
V D R T G K V K S L P G C E T S Q I					
2332	2341	2350	2359	2368	2377
TTG GAG GGA AGA TAA TAG GTA CAT TCA CCG GCG TTC AAG AAA ATC TCA CTA TCT					
---	---	---	---	---	---
L E G R *					
2386	2395	2404	2413	2422	2431
GAT CAA GGG AAT TCC GCC TAA TGT AGC TTT TGA AAA TTT TCA AGA ATC CTC ATT					
---	---	---	---	---	---
2440	2449	2458	2467	2476	2485
TCT ACA GTT TGA GAG TAT CTT TTT TGT ATT GTT TCT GCA CCA TGT TTC AGA TAG					
---	---	---	---	---	---
2494	2503	2512	2521	2530	2539
GCC ATA ATT TCC AGC TTA TAA TCC CAT TAT GGG TAG ATT TTC CTA TTC TTT TCT					
---	---	---	---	---	---
2548	2557	2566	2575	2584	2593
CCT ATT TTT TGC CTA GCG TTG GTT TAC AAA CTA GAG TGA GAA AAG GTT TCA ATG					
---	---	---	---	---	---
2602	2611	2620	2629		
TAT TCT GAT GCT TTT GAT TAG AAA TGC AAT AAC ATT GGT TAA AA 3'					
---	---	---	---		

Figure 3. The DNA sequence of the putative PLD clone Nt10B1 and the corresponding amino acid sequence. Nt10B1 was obtained from a screen of the tobacco cell culture cDNA library using the partial PLD clone (AF195614) as a probe. The sequence homologous to other PLDs begins 599 base pairs into the clone (Ⓢ). The 5' end of the clone contains a region that has homology to the sequence of the plant histone H2B. The green arrows (→) mark the primers used to confirm the transgenic identity of the PLD antisensed tobacco plants. The pink arrow (→) marks the primer used to PCR amplify sequences subcloned into pTrcHis and pTrcHis2 to study protein expression.

Clone Nt10B1	ARRLIYITGW	SVYHLVTLVR	DNG-KAEESM	LGEILKRKSO	EGVRVLLLIW
<i>Lycopersicon</i> β1	ARRLIYITGW	SVYHLVTLVR	DN-DNAEKSM	LGEILKRKSO	EGVRVLLLIW
<i>Arabidopsis</i> β1	ARRLIYITGW	SVVHKVKLIR	DKLGPASECT	LGELLRSKSO	EGVRVLLSIW
<i>Lycopersicon</i> β2	ARRLIYITGW	SVVHKVKLVR	DD-ASAEGCT	LGDLLKLKSO	EGVRVLLLIW
<i>Arabidopsis</i> β2	ARRLIYITGW	SVVHNVRLVR	DKEDESSECR	LGELLRSKSO	EGVRVLLLVW
<i>Arabidopsis</i> γ1	GRRLIYITGW	SVFHPVRLVR	RTNDPTEG-T	LGELLKVKSO	ELECW--FW
<i>Arabidopsis</i> γ2	ARRLIYITGW	SVFHPVRLVR	RNNDPTEG-T	LGELLKVKSO	EGVRVL--GFG
Clone Nt10B1	DDPTS-SKIL	GYKSEITMGT	SDEETRRYFK	HSSVHVLLCP	RSAGKGHSWV
<i>Lycopersicon</i> β1	DDPTS-KSIL	GYKTEITMGT	NDEETRRYFK	HSSVQVLLCP	RSAGKGHSWA
<i>Arabidopsis</i> β1	DDPTS-RSIL	GYKTDGVMAT	HDEETRRFFK	HSSVQVLLCP	RNAGKRHSWV
<i>Lycopersicon</i> β2	DDPTS-RSIL	GYKTDGVMAT	HDEETRSFFK	HSSVKVLLCP	RVAGKRHSWV
<i>Arabidopsis</i> β2	DDPTS-QNIL	GYMTDGVMGT	HDEETRRFFK	DSSVQVLLCP	RNAGKRHSWV
<i>Arabidopsis</i> γ1	CGTIQGV-FW	DSKTQGVMT	SDEETRRFFK	HSSVQVLLCP	RSGGKGHSFI
<i>Arabidopsis</i> γ2	VDDPTSMSFP	GFSIKGLMNT	SDEETRRFFK	HSSVQVLLCP	RYGGKGHSFI
Clone Nt10B1	KKQETGTIYT	HHQKTIVLDV	DAGNYQRKII	AFVGGDLCLK	GRYDTPQHPI
<i>Lycopersicon</i> β1	KKQETETIYT	HHQKTIVLDA	DAGNYQRKIM	AFVGGDLCLK	GRYDTPAHPI
<i>Arabidopsis</i> β1	KQREVGTIYT	HHQKNVIVDA	DAGGNRRKII	AFVGGDLCLD	GRYDTPQHPL
<i>Lycopersicon</i> β2	KQREVGVIYT	HHQKTIVLDA	DAGNNRRKIV	AFVGGDLCLD	GRYDTPQHPL
<i>Arabidopsis</i> β2	KQREVGTIYT	HHQKNLIVDA	DAGGNRRKIV	AFVGGDLCLD	GRYDTPQHPL
<i>Arabidopsis</i> γ1	KKSEVGTIYT	HHKKTIVVDS	EAAQNRKIV	AFVGGDLCLN	GRFDTPKHPL
<i>Arabidopsis</i> γ2	KKSEVEETIYT	HHQKTIVLDA	EAAQNRKIV	AFVGGDLCLN	GRFDTPKHSL
Clone Nt10B1	EKTLQNVHKD	DYHQPNTGTP	TTGCPREPWH	DLHSRIECPA	AYDVLTNFEE
<i>Lycopersicon</i> β1	FRTLQNVHKD	DFHQPNTGTP	TTGCPREPWH	DLHSRIECPA	AYDVLTNFEE
<i>Arabidopsis</i> β1	FRTLQTIHID	DFHNPFTFTGN	LSGCPREPWH	DLHSDKIDGPA	AYDVLTNFEE
<i>Lycopersicon</i> β2	FRTLKTVHSE	DYHNPETYAGS	TAGCPREPWH	DLHSDKIDGPA	AYDVLTNFEE
<i>Arabidopsis</i> β2	FRTLQTDHNG	DYHNPFTFTGN	VS GCPREPWH	DLHSDKIDGPA	AYDVLTNFEE
<i>Arabidopsis</i> γ1	FRTLKTLHKD	DFHNPNEVTT	ADDGPREPWH	DLHSDKIDGPA	AYDVLANFEE
<i>Arabidopsis</i> γ2	FRTLKTLHKD	DFHNPNEVTT	EDVGPREPWH	DLHSDKIDGPA	AYDVLANFEE
Clone Nt10B1	RWLKASKRHG	LQKMAASHDD	ALLQLDRIPD	ILKLIADVPC	GEDDADTWHV
<i>Lycopersicon</i> β1	RWLKASKRHG	LQKMAASHDD	ALLKIDRIPD	ILGIADVPC	REDDADTWHV
<i>Arabidopsis</i> β1	RWLKAAKPSG	IKKFKLPIDD	ALLRIDRIPD	ILGVSDTPTV	SENDPEAWHV
<i>Lycopersicon</i> β2	RWLKASKPHG	IRKLKTSFED	DLRLBEMFE	IVGISDAPSV	SSDDPNGWHV
<i>Arabidopsis</i> β2	RWLKAAKPHR	INKLKTSYDD	ALLRIDRIPD	IIRVLDAPT-	-----
<i>Arabidopsis</i> γ1	RWMKASKLAE	LGNENTSSDD	SLLRIDRIPD	IVGLSEASSA	NDNDPESWHV
<i>Arabidopsis</i> γ2	RWMRLQNLRG	IGEGRTSFDD	SLLRIDRIPD	IMGLSEASSA	NDNDPESWHV
Clone Nt10B1	QIFRSIDSNS	VKGFPKDPKE	ATNKNLVCGK	NVLIDMSIHT	AYVKAIRAAQ
<i>Lycopersicon</i> β1	QIFRSIDSNS	VKGFPKDPKE	ATNMNLVCGK	NVLIDMSIHT	AYVKAIRAAQ
<i>Arabidopsis</i> β1	QIFRSIDSNS	VKGFPKDPKD	ATCKNLVCGK	NVLIDMSIHT	AYVKAIRAAQ
<i>Lycopersicon</i> β2	QIFRSIDSNS	VKGFPKDPKE	ATMKNLVCGK	NVLIDMSIHT	AYVKAIRAAQ
<i>Arabidopsis</i> β2	-IFRSIDSNS	VKGFPKDPKY	ATSKNLVCGK	NVLIDMSIHT	AYVKAIRAAQ
<i>Arabidopsis</i> γ1	QVFRSIDSNS	VKGFPKDPKE	ATGRNLVCGK	NILIDMSIHA	AYVKAIRSAQ
<i>Arabidopsis</i> γ2	QVFRSIDSNS	VKGFPKDPKE	ATGRNLVCGK	NILIDMSIHA	AYVKAIRSAQ
Clone Nt10B1	HFIYIENQYF	LGSSYNWNNY	QDLGANNLIP	MEIALKIANK	IRANERFSVY
<i>Lycopersicon</i> β1	HFIYIENQYF	LGSSYNWSNY	QNLGANNLIP	MEIALKIANK	IRANERFAAY
<i>Arabidopsis</i> β1	HFIYIENQYF	IGSSYNWNAH	KDIGADNLIP	MEIALKIAEK	IRANERFAAY

Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

Clone Nt10B1
Lycopersicon $\beta 1$
Arabidopsis $\beta 1$
Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

Clone Nt10B1
Lycopersicon $\beta 1$
Arabidopsis $\beta 1$
Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

Clone Nt10B1
Lycopersicon $\beta 1$
Arabidopsis $\beta 1$
Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

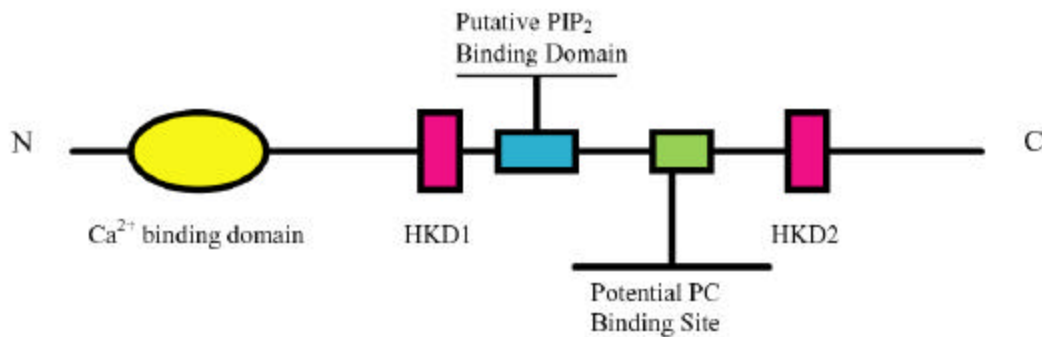
Clone Nt10B1
Lycopersicon $\beta 1$
Arabidopsis $\beta 1$
Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

Clone Nt10B1
Lycopersicon $\beta 1$
Arabidopsis $\beta 1$
Lycopersicon $\beta 2$
Arabidopsis $\beta 2$
Arabidopsis $\gamma 1$
Arabidopsis $\gamma 2$

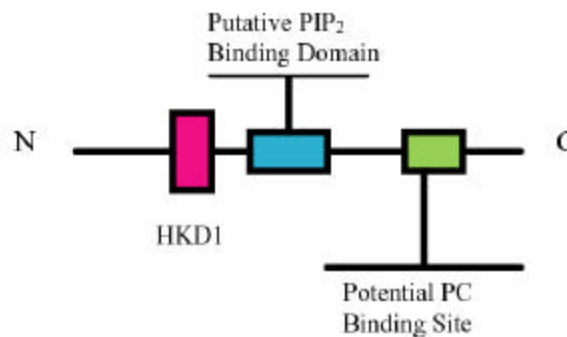
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HFIYIENQYF	IGSSYDWNAAH	KDIGANNLIP	MEIALKIAEK	IRAKERFAAY
HFIYIENQYF	LGSSFNWDSN	KDLGANNLIP	MEIALKIANK	IRAREKFAAY
HFIYIENQYF	LGSSFNWDSN	KNLGANNLIP	MEIALKIANK	IRAREKFAAY
360	370	380	390	400
IVPMWPEGV	PTSTATORIL	FWQHNTMOMM	YETIYKALVE	VGLENTYEPO
IVLPMWPEGN	PTSTPTQRIIL	FWQYNMOMM	YETIYKALVE	VGLENTYEPO
IVIPMWPEGV	PTGAATORIL	YWQHKTIMM	YETIYKALVE	TGLEGAFSPQ
IVLPMWPEGN	PTGAATORIL	YWQNKTMOMM	YETIYKALVE	VGLENSCSPQ
IVIPMWPEGV	PTGAATORIL	YWQHKTIMM	YGTIYNALVE	AGLEDEYSPO
IVIPMWPEGA	PTSNPIORIL	YWQHKTIMM	YQTIYKALVE	VGLDSQFEPO
IVIPMWPEGA	PTSNPIORIL	YWQHKTIMM	YQTIYKALVE	VGLDGQLEPO
410	420	430	440	450
DYLNFFCLGN	REVQ-----	-----ED	GN-----NT	VVKSSKPTTP
DYLMFFCLGN	REVPE----N	GITTIVRS--	-----	----SKPNTTP
DYLNFFCLGN	REMYDGIDNS	GTGS-----	----PRQMCIT	LLRP-----
DYLNHYCLGN	RE----AGKVE	GNES-----	----P-----	----SAANT
DYLNFFCLGN	REMY-----	----NGNNES	GTGS-----	----ASNENT
DFLNFFCLGT	REV-----PV	GTVSVYNSPR	KPPQPN----	----ANANAA
DFLNFFCLGT	REVGTRVID	GTVSVYNSPR	KPPQ-----	-----LNAA
460	470	480	490	500
QE--LSQKSR	RFMIYVHSGK	MIVDDEYVIL	GSANINQRSL	EGTRDTEIAM
--QEITQKSR	RFMIYVHSGK	MIVDDEYVIM	GSANINQRSL	EGTRDTEIAM
-L---SRKSR	RFMIYVHSGK	MVVDDEYVVI	GSANINQRSM	EGTRDTEIAM
P-QAFSOKSR	RFMIYVHSGK	MIVDDEYVIL	GSANINQRSL	EGTRDTEIAM
P-QGLCRKSR	RFMIYVHSGK	MVVDDEYVVI	GSANINQRSM	EGTRDTEIAM
QVQAL--KSR	RFMIYVHSGK	MVVDDEYVLI	GSANINQRSL	EGTRDTEIAM
QVQAL--KSR	RFMIYVHSGK	MVVDDEYVLI	GSANINQRSL	EGTRDTEIAM
510	520	530	540	550
GSYOPHHTWA	TKHSRPHGQV	YGYRMSLWAE	HTGTLEOCFE	HPESLECVR
GAYOPHHTWA	NKHSCEHAQV	YGYRMSLWAE	HTGTLEOCFE	HPESLECVR
GAYOPHTWA	RKHSCEPRGOI	YGYRMSLWAE	HMANLDDCF	QPESECVRK
GAYOPHHTWA	RKOSTEYGOI	HGYRMSLWAE	HLGVVEDCF	QPESECVRR
GAYOPHTWA	RRQSGPRGOI	YGYRMSLWAE	HMANLDDCF	EPESLGCVRK
GGYOPHYSWA	MKGSRMLMVR	W-YRMSLWAE	HLGFLEOCFE	EPENMECVRR
GGYOPHHSWA	KKGSRPRGOI	FGYRMSLWAE	HLGFLEOCFE	EPENMECVRR
560	570	580	590	600
IVVFGHEHNWL	QYAADEVTEM	RGHLLKYPVE	VDRTGKVKSL	PGCET-----
IVVFGHEHNWL	QYAADEVTEM	KGHLLKYPVE	VDRTGKVRSL	PGCETFPYWR
VRTMGHERNWK	QFAAEVSDM	RGHLLKYPVE	VDRKGKVRPL	PGSETFPD-V
VRSMGEYNNWK	QFASDEVTEM	RGHLLKYPVE	VDRKGKVKNL	TGCANFPD-V
VRTVALENWE	QFRSEVSEEM	RGHLLKYPVE	VDRKGKVRPL	PGSEEFDPD-V
VRQLSELNWR	QYAADEVTEM	SGHLLKYPVQ	VDRTGKVSLL	PGCETFPD-L
VRQLSELNWR	QYAADEVTEM	PGHLLKYPVQ	VDRTGKVSLL	PGYETFPD-L

Figure 4. Alignment of the predicted amino acid sequence of Nt10B1 (Accession No. AY138861) respectively with PLD β 1 from *Lycopersicon esculentum* (Protein Accession No.AAG45487) and *Arabidopsis thaliana* (Protein Accession No.AAB63542), PLD β 2 from *Lycopersicon esculentum* (Protein Accession No.AAG45488) and *Arabidopsis thaliana* (Protein Accession No.AAF02803) and PLD γ 1 (Protein Accession No.CAB78228) and γ 2 (Protein Accession No.CAB78226) from *Arabidopsis thaliana*. The conserved amino acids are highlighted in black. Nt10B1 shares 92% identity with PLD β 1 from *Lycopersicon esculentum*, 76% identity with PLD β 2 from *Lycopersicon esculentum*, 76% identity with PLD β 1 from *Arabidopsis thaliana*, 72% identity with PLD β 2 from *Arabidopsis thaliana*, 70% identity with PLD γ 1 from *Arabidopsis thaliana*, 68% identity with PLD γ 2 from *Arabidopsis thaliana*. All identities were deduced at the amino acid level. Sequences were aligned using DNASIS V2.1.

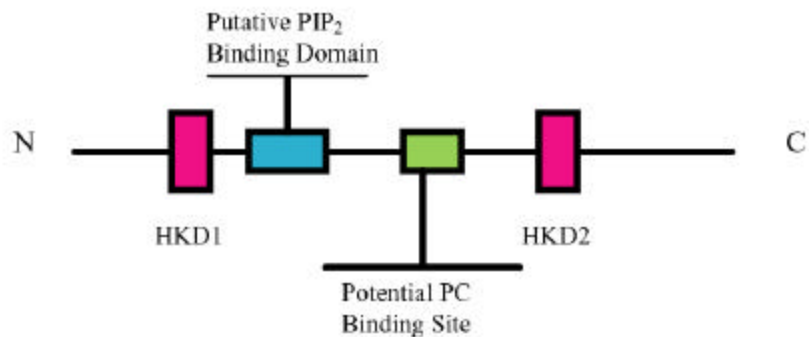
Arabidopsis thaliana PLDb1 Domains



Partial PLD AF195614 used to screen tobacco cell culture cDNA library



Tobacco Clone Nt10B1: a PLDb1



1	ARRLIYITGWSVYHLVTLVRDNGKAEESMLGEILKRKSQEGVRVLLLIWDDPTSSKSILG	60
61	YKSEGIMGTSDEETRRYFKHSSVHLLCPRSAGKGHSWVKKQETGTIYTHHQTIVIVD	120
121	AGNYQRKIIAFVGGDLCKGRYDTPQHPIFKTLQNVHKDDYHQPNYTGPTTGCPREPWH	180
181	LHSRIEGPAAYDVLTNFEEHLWKASKRHGLQMKASQDDALLQDRIPDILKIADVPC	240
241	EDDADTWHVQIFRSIDSNVKGFPKDPKEATNKNLVCCKNVLDMSIHTAYVKAIRAAQH	300
301	FLYIENQYELGSSYNWNNYQDLGANNLIPMEIALKIANKIRANERFSVYIIVPMWPEGVP	360
361	TSTATQRILFWQHNTMQMMYETIYKALVEVGLENTYEPQDYLNFFCLGNREVQEDGNNTV	420
421	VKSSKPTTPQELSQKSRRFMIYVHSGMIVDDEYVILGSANINQRSLEGTRDTEIAMGSY	480
481	QPHHTWATKHSRPHGQVYGYRMSLWAEHTGTLEQCFEHPESLECVRRIRVFGHNWLQYA	540
541	ADEVTEMRGHLKYPVEVDRTGKVKSLPGCETSQILEGR*	580

Figure 5. Plant PLDs, including *Arabidopsis thaliana* PLD β 1 (shown here as PLDb1) (Protein Accession No. AAB63542), are known to have certain conserved regions such as the C2 calcium-binding domain, the two catalytic domains (HKD1 and HKD2), and the putative PIP₂ and PC binding sites (redrawn from Qin and Wang, 2002). The partial PLD clone AF195614 was used to screen a tobacco cell culture cDNA library. The resulting clone is Nt10B1 (Accession No. AY138861). Shown here is the amino acid sequence. The methionine residue shown in red (M) marks the beginning of the “new” sequence that is additional to the previously cloned PLD fragment (AF195614).

<u>1st HKD Motif</u>	---	H	x	K	x	x	x	x	D	---
Clone Nt10B1	---	H	Q	K	T	V	I	V	D	---
<i>Lycopersicon esculentum</i> PLDβ1	---	H	Q	K	T	V	I	L	D	---
<i>Arabidopsis thaliana</i> PLDβ1	---	H	Q	K	N	V	I	V	D	---
<i>Lycopersicon esculentum</i> PLDβ2	---	H	Q	K	T	V	I	I	D	---
<i>Arabidopsis thaliana</i> PLDβ2	---	H	Q	K	N	L	I	V	D	---
<i>Arabidopsis thaliana</i> PLDγ1	---	H	E	K	T	V	I	V	D	---
<i>Arabidopsis thaliana</i> PLDγ2	---	H	Q	K	T	M	I	V	D	---

<u>2nd HKD Motif</u>	H	x	K	x	x	x	x	D	xxxxxx	G	S	A	N	I	N	Q	R
Clone Nt10B1	H	S	K	G	M	I	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>L.e.</i> PLDβ1	H	S	K	G	M	I	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>A.t.</i> PLDβ1	H	S	K	G	M	V	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>L.e.</i> PLDβ2	H	S	K	G	M	I	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>A.t.</i> PLDβ2	H	S	K	G	M	V	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>A.t.</i> PLDγ1	H	S	K	G	M	V	V	D	xxxxxx	G	S	A	N	I	N	Q	R
<i>A.t.</i> PLDγ2	H	S	K	G	M	V	V	D	xxxxxx	G	S	A	N	I	N	Q	R

<u>IYIENQYF Motif</u>	---	I	Y	I	E	N	Q	Y	F	---
Clone Nt10B1	---	I	Y	I	E	N	Q	Y	F	---
<i>Lycopersicon esculentum</i> PLDβ1	---	I	Y	I	E	N	Q	Y	F	---
<i>Arabidopsis thaliana</i> PLDβ1	---	I	Y	I	E	N	Q	Y	F	---
<i>Lycopersicon esculentum</i> PLDβ2	---	V	Y	I	E	N	Q	Y	F	---
<i>Arabidopsis thaliana</i> PLDβ2	---	I	Y	I	E	N	Q	Y	F	---
<i>Arabidopsis thaliana</i> PLDγ1	---	I	Y	I	E	N	Q	Y	F	---
<i>Arabidopsis thaliana</i> PLDγ2	---	I	Y	I	E	N	Q	Y	F	---

<u>Putative PIP₂ Binding Motif</u>	R	x	x	x	x	x	K	x	x	x	x	x	K
Clone Nt10B1	R	W	L	K	A	S	K	R	H	G	L	Q	K
<i>Lycopersicon esculentum</i> PLDβ1	R	W	L	K	A	S	K	R	H	G	L	Q	K
<i>Arabidopsis thaliana</i> PLDβ1	R	W	L	K	A	A	K	P	S	G	I	K	K
<i>Lycopersicon esculentum</i> PLDβ2	R	W	L	K	A	S	K	P	H	G	I	R	K
<i>Arabidopsis thaliana</i> PLDβ2	R	W	L	K	A	A	K	P	H	R	I	N	K
<i>Arabidopsis thaliana</i> PLDγ1	R	W	M	K	A	S	K	L	A	E	L	G	N
<i>Arabidopsis thaliana</i> PLDγ2	R	W	M	R	L	S	L	N	R	G	I	G	E

Figure 6. Alignment of the predicted protein domains of Nt10B1 (Accession No. AY138861) respectively with predicted protein domains of PLD β 1 from *Lycopersicon esculentum* (Protein Accession No.AAG45487) and *Arabidopsis thaliana* (Protein Accession No.AAB63542), PLD β 2 from *Lycopersicon esculentum* (Protein Accession No.AAG45488) and *Arabidopsis thaliana* (Protein Accession No.AAF02803) and PLD γ 1 (Protein Accession No.CAB78228) and γ 2 (Protein Accession No.CAB78226) from *Arabidopsis thaliana*. The general motif identified in *Arabidopsis* PLDs is given first in each case.

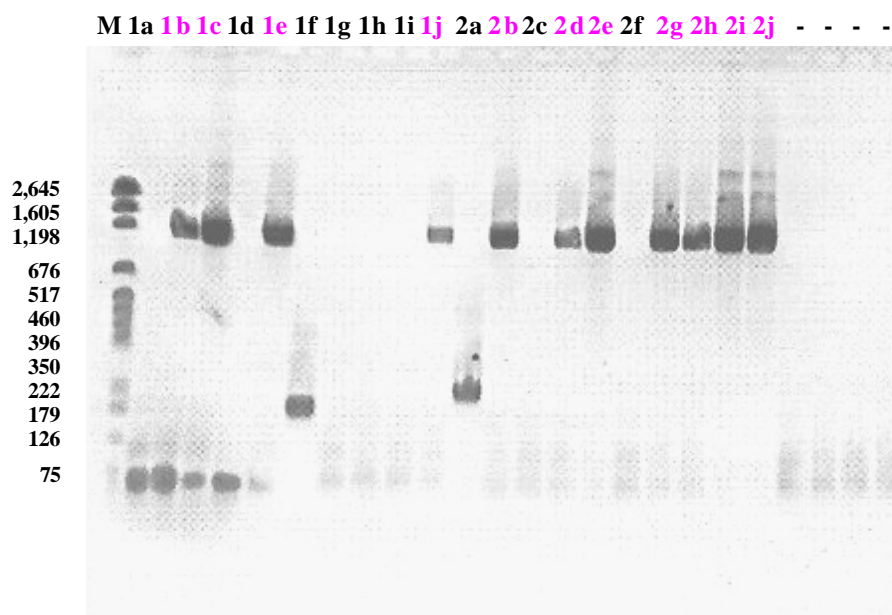
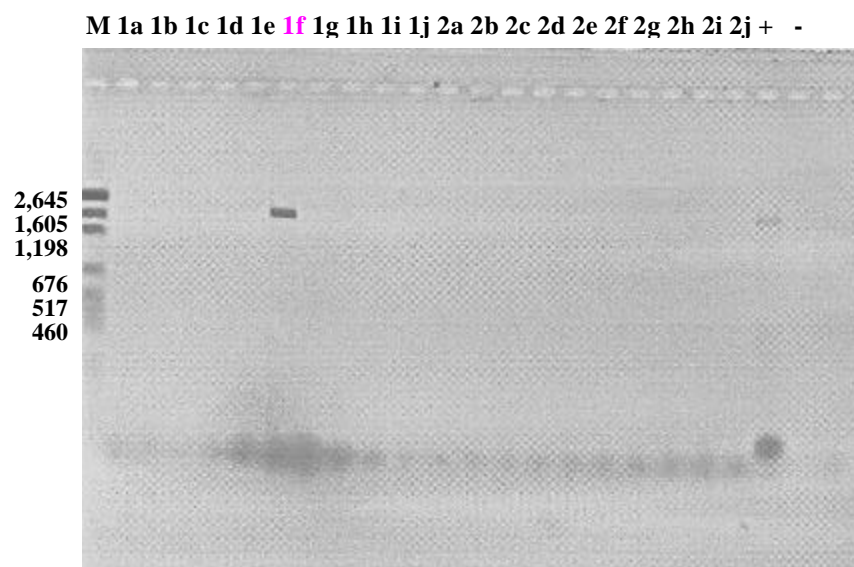


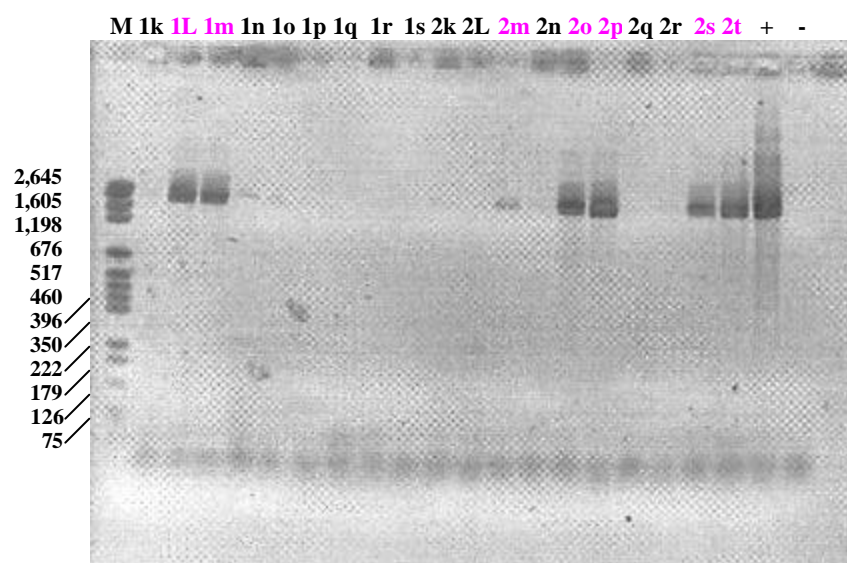
Figure 7. Screening post-transformation Express1.2 colonies by PCR to identify colonies with an insert of the correct size. Single colonies were selected and touched with a sterile loop, which was briefly dipped in 50 μ l sterile milliQ water for colony PCR screening. The same loop was then used to streak the colony (first streak only) on a fresh LB plate and to inoculate liquid cultures. Ten colonies were selected in this manner per transformation (1a-1j and 2a-2j). The 50 μ l sample containing the bacterial cells was boiled for 5 min to lyse cells. The sample was then used as template for a PCR reaction to determine whether the colony contained the vector with an insert of the correct size. The PCR reaction primers (in this case ExpresF2 and PLDR) and conditions were identical to those used to generate the insert for ligation into pTrcHis and pTrcHis2 with the exception of the template. Products were visualized on a 2% agarose gel after staining with ethidium bromide. Numbers shown in color indicate colonies selected for plasmid

extraction and sequencing of the insert. 1a-1j: PCR screen of colonies resulting from the ligation of Expres1.2 and transformed into pTrcHis; 2a-2j: PCR screen of colonies resulting from the ligation of Expres1.2 and transformed into pTrcHis2; ‘-’: negative controls, where the template for the reaction was generated by touching the bacterial plate agar not touching a colony, dipping the loop in water and boiling for 5 min.

A



B



C

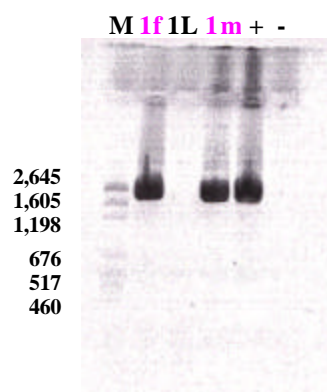


Figure 8. Screening post-transformation Expres1.8 colonies by PCR to identify colonies with an insert of the correct size. Single colonies were selected and touched with a sterile loop, which was briefly dipped in 50 μ l sterile milliQ water for colony PCR screening. The same loop was then used to streak the colony (first streak only) on a fresh LB plate and to inoculate liquid cultures. Ten colonies were selected in this manner per transformation (1a-1j, 2a-2j, 1k-1s and 2k-2t). The 50 μ l sample containing the bacterial cells was boiled for 5 min to lyse cells. The sample was then used as template for a PCR reaction to determine whether the colony contained the vector with an insert of the correct size. The PCR reaction primers (in this case ExpresF2 and the universal T7 primer) and conditions were identical to those used to generate the insert for ligation into pTrcHis and pTrcHis2 with the exception of the template. Products were visualized on a 2% agarose gel after staining with ethidium bromide. Numbers shown in color indicate colonies selected for plasmid extraction and sequencing of the insert. (A) 1a-1j: PCR screen of colonies resulting from the ligation of Expres1.8 and transformed into pTrcHis; 2a-2j: PCR screen of colonies resulting from the ligation of Expres1.8 and transformed into pTrcHis2; '+': positive control using Nt10B1 in pBluescript as template; '-': negative control, where the template for the reaction was generated by touching the bacterial plate agar not touching a colony, dipping the loop in water and boiling for 5 min. (B) 1k-1s: PCR screen of colonies resulting from the ligation of Expres1.8 and transformed into pTrcHis; 2k-2t: PCR screen of colonies resulting from the ligation of Expres1.8 and transformed into pTrcHis2; '+': positive control using Nt10B1 in pBluescript as template; '-': negative control, where the template for the reaction was

generated by touching the bacterial plate agar not touching a colony, dipping the loop in water and boiling for 5 min. (C) In the case of cloning the 1.8 kb fragment into pTrcHis, the pTrcHis Xpress™ Forward primer was used along with the ExpresF2 primer to determine if the insert had been inserted in the wrong direction. 1f and 1m: insert in the wrong direction; 1L: insert in the correct direction; '+': positive control using Nt10B1 in pBluescript as template; '-': negative control with no template

Six clones were selected for subsequent studies. These included Expres1.8 in pTrcHis (Expres1.8HIS) and Expres1.8 in pTrcHis2 (Expres1.8HIS2). Both clones contained the full subcloned region of Nt10B1, however, Expres1.8HIS is in frame with the HIS tag (multiple histidine residues) and will therefore be expressed as a fusion peptide (Figure 13A). Expres1.8HIS2 has a stop codon upstream of the HIS tag encoding sequence and therefore will not be translated with the HIS tag attached. Two clones also were chosen that contained the coding sequence in reverse orientation as controls. Finally, Expres1.2 in pTrcHis (Expres1.2HIS) and Expres1.2 in pTrcHis2 (Expres1.2HIS2) were selected. Expres1.2HIS was constructed to contain the His tag as a fusion peptide at the amino terminus while Expres1.2HIS2 contained it at the carbonyl terminus (Figure 13A).

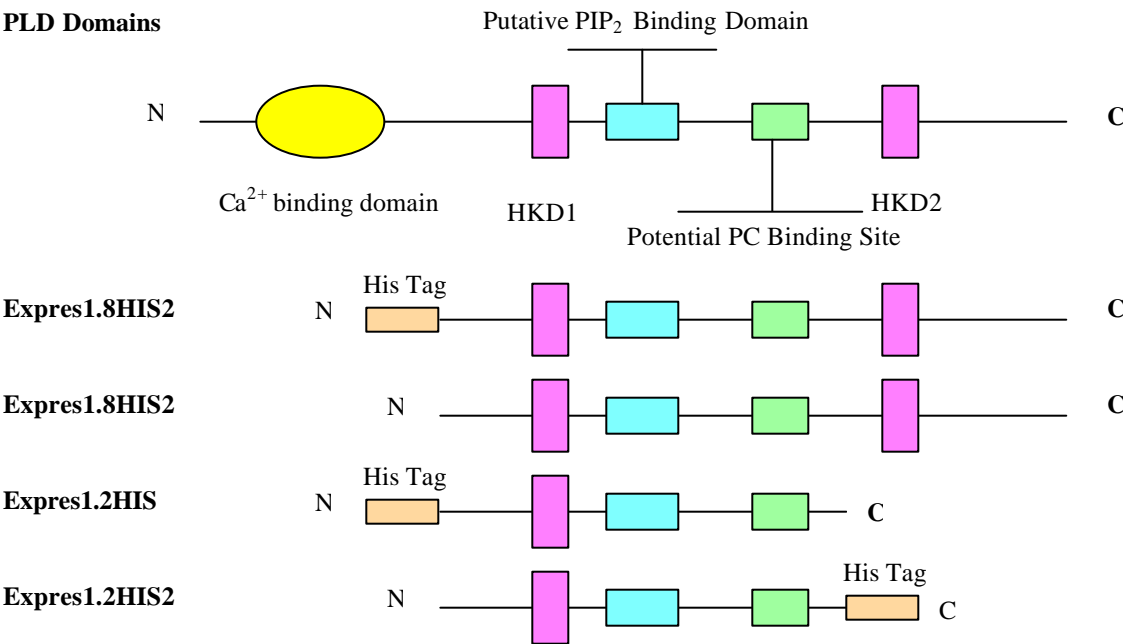
Expression and Transphosphatidylation Activity of the Clones

The transformants containing the clones described above were grown overnight with 1 mM IPTG to induce gene expression as were non-transformed Top10 cells and cells carrying the recombinant *Arabidopsis* PLD β and ? in pBluescript. Total protein was extracted and 20 μ g was assayed for transphosphatidylation activity towards radiolabelled PC in the presence of ethanol. Activity was determined by quantification of radiolabelled phosphatidylethanol. The Expres clones showed no PLD activity. Expres1.8His and PLD β were assayed in three different conditions: 1 M EGTA, 50 μ M CaCl $_2$ and 25 mM CaCl $_2$. PLD β was optimally active in micromolar calcium conditions as previously described (Pappan *et al.*, 1998) Expres1.8His was inactive throughout the range of conditions.

Protein Isolation and SDS PAGE

The His tag is designed to enable purification of the protein of interest. In this case we used the ProBond system to take advantage of the multiple histidine residues. Using this system we were able to isolate Expres1.8HIS and Expres1.2HIS. When separated by SDS PAGE, Expres1.8HIS was found to have a molecular weight of 74 kDa and Expres1.2HIS, 48 kDa (Figure 13C).

A



B

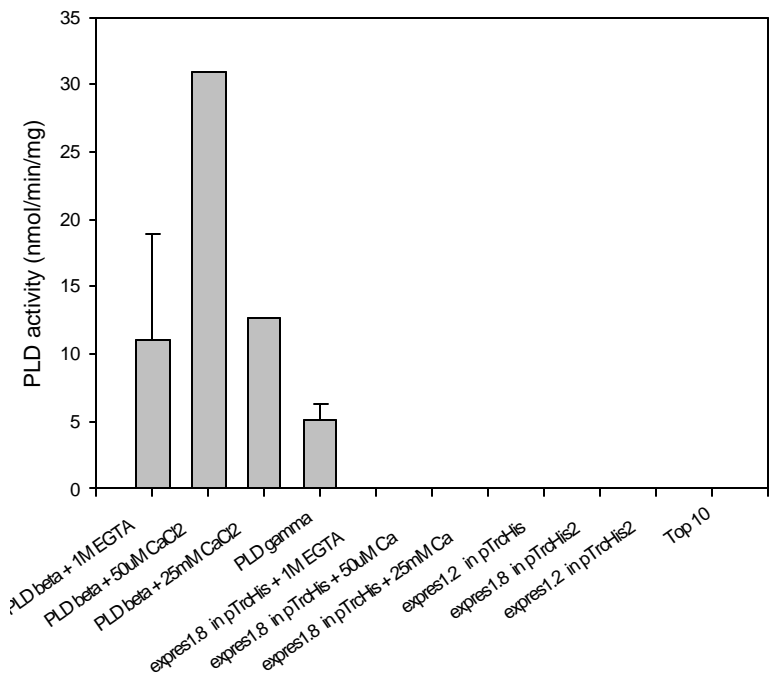


Figure 9. Protein expression. Specific regions of Nt10B1 were PCR amplified and cloned into pTrcHis and pTrcHis2 to study protein expression. (A) A primer was designed (expresF2) at the 5' end of the subsequence of NT10B1 (the region homologous to other plant PLDs) for PCR amplification (see Figure 8). The 3' primer used was either the universal T7 primer or the PLDR primer. The resulting products were respectively 1.8 kb in length (amplified from Nt10B1 base pair 601 to 2640) and 1.2 kb length (amplified from Nt10B1 base pair 601 to 1690) and respectively named Expres1.2 and Expres1.8. Both fragments were ligated into the expression vectors pTrcHis and pTrcHis2. Clones included Expres1.8 in pTrcHis (Expres1.8HIS), Expres1.8 in pTrcHis2 (Expres1.8HIS2), Expres1.2 in pTrcHis (Expres1.2HIS) and Expres1.2 in pTrcHis2 (Expres1.2HIS2). Expres1.8HIS, Expres1.2HIS and Expres1.2HIS2 are in frame with the HIS tag (multiple histidine residues) and will therefore be expressed as a fusion peptides. Expres1.8HIS and Expres1.2HIS were constructed to contain the His tag as a fusion peptide at the amino terminus while Expres1.2HIS2 contains it at the carbonyl terminus. Expres1.8HIS2 has a stop codon upstream of the HIS tag encoding sequence and therefore will not be translated with the HIS tag attached providing a useful control. Expres1.8HIS and Expres1.8HIS2 contain all the protein motifs found in Nt10B1 whereas Expres1.2HIS and Expres1.HIS2 lack the second *HKD* domain required for catalysis. All clones lack the calcium-binding domain usually found in PLDs. (B) Transphosphatidylase activity of the clones. Cells were grown overnight with 1 mM IPTG to induce gene expression as were control non-transformed Top10 cells and cells carrying the recombinant *Arabidopsis* PLD β and ?. Total protein was extracted and 20 μ g was assayed for

transphosphatidylation activity towards radiolabelled PC in the presence of ethanol.

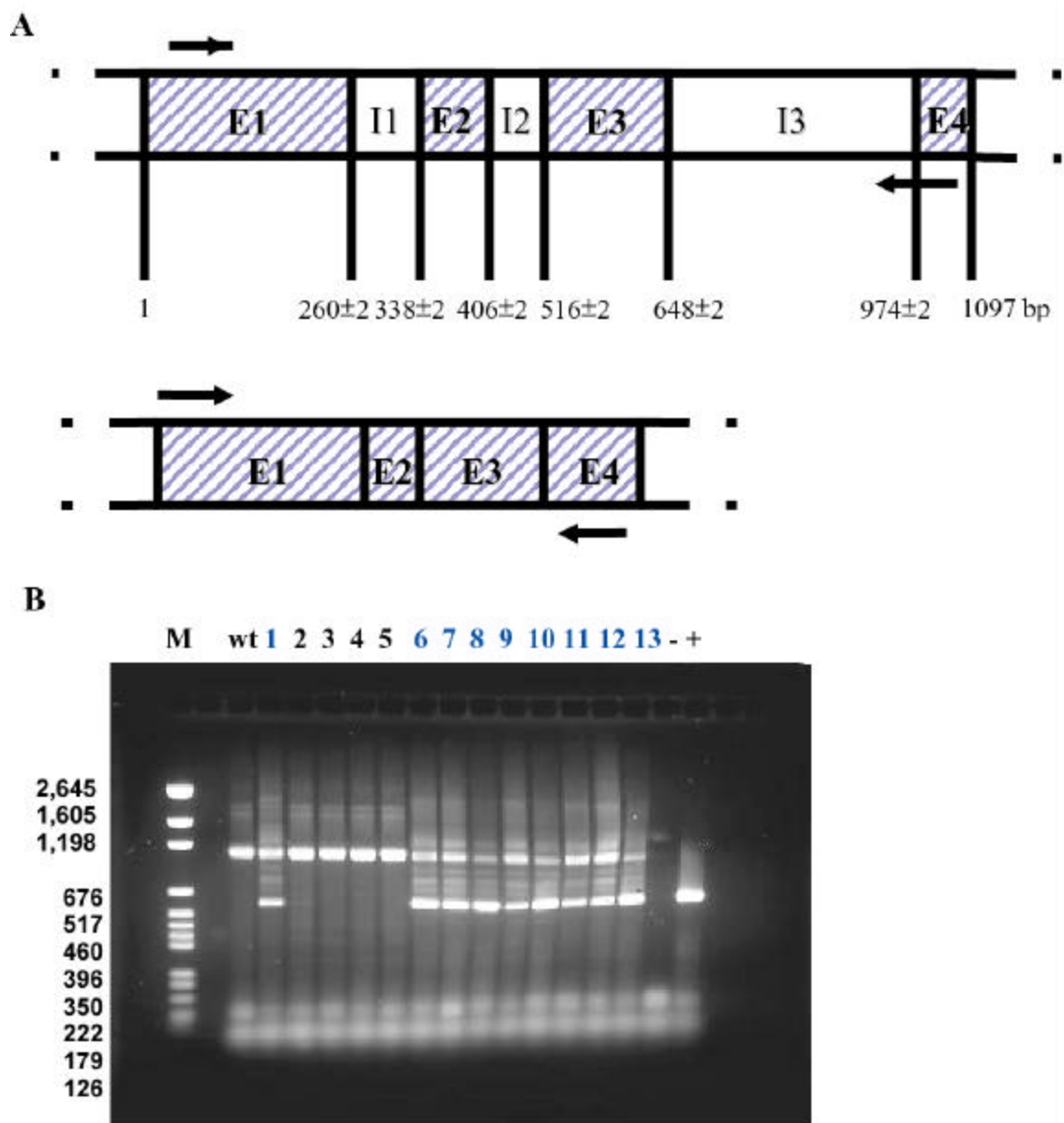
Activity was determined by quantification of radiolabelled phosphatidylethanol.

Expres1.8His and PLD β were assayed in three different conditions: 1 mM EGTA, 50 μ M CaCl_2 and 25 mM CaCl_2 . These data are the result of one experiment. Each assay was performed in triplicates. In samples showing no standard deviation, only duplicate data was available.

Identification of Transgenic Tobacco Plants

To manipulate the expression of PLD, transgenic tobacco plants (cv xanthi) were generated (by Dr. Swati Tripathy) harboring a partial PLD cDNA clone previously isolated in this lab (AF195614) (by Shea Austin-Brown) in reverse orientation under the regulation of the CaMV 35S promoter (Figure 2). A PCR strategy was used to confirm that the transgene had been incorporated into the tobacco genome of the mature plants originally selected on kanamycin. DNA was extracted from the mature transgenic tobacco plants and wild type plants. The genomic DNA was subjected to PCR amplification with primers designed to amplify a 601 bp region of the antisense cDNA fragment (PLDF and PLDR). Plasmid DNA (the partial PLD cDNA clone ligated into pZero) was used as a positive control. In most of the transgenic plant samples a higher molecular weight band around 1.1 kb was observed in addition to the 601 bp product. In addition the 1.1 kb product was the only band found in the wild type sample (Figure 10B). We hypothesized that the larger PCR product was the result of the PCR amplification of the endogenous gene and that the difference in size was due to the presence of naturally occurring introns. To confirm this hypothesis, DNA from wild type tobacco was used as template in a PCR reaction to generate a blunt-ended fragment using the PLDF and R primers. The band was gel purified and cloned into the vector pZero. Three clones were completely sequenced. By comparing the sequence generated (Accession No. AY138862) to the known sequence of the partial PLD cDNA clone we were able to determine that the region of the endogenous gene amplified by the PLDF and PLDR primers contained three introns (Figure 3A). We were therefore able to

confirm from our previous PCR results the insertion of the transgene into the transgenic plants ASPLD1, 6, 7, 8, 9, 10, 11, 12, and 13 (Figure3B).



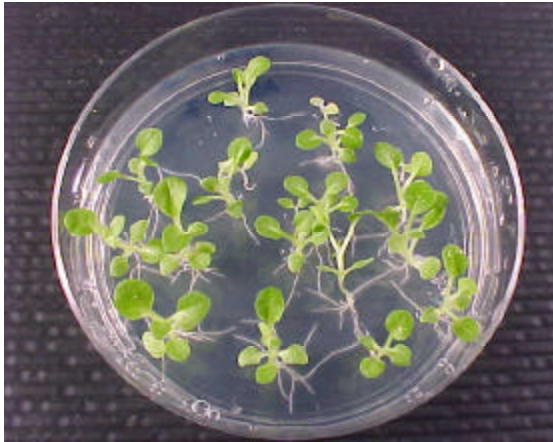
C

1	CCTAACTACACGGGGCCCTACTACCGGTTGTCCTAGAGAACCTTGGCATGATTTACATAGT	60
1	P N Y T G P T T G C P R E P W H D L H S	20
61	CGGATCGAGGGGCTGCTGCATATGATGTCCTAACTAACTTCGAGGAGCGCTGGTTGAAG	120
21	R I E G P A A Y D V L T N F E E R W L K	40
121	GCTTCAAAGCGCCATGGACTTCAAAAGATGAAAGCTTCACAAGATGATGCATTACTCCAA	180
41	A S K R H G L Q K M K A S Q D D A L L Q	60
181	CTTGACAGGATTCCCGACATATTA AAAATAGCTGATGTCCCTTGCCTAGGAGAAGATGAT	240
61	L D R I P D I L K I A D V P C L G E D D	80
241	GCAGATACGTGGCACGTGCAGGTACTTAAAAGTTGAATCTAGCATTGCTTGTTAAGTTCC	300
81	A D T W H V Q	
	Intron 1	
301	CTTTTGTTCCTCTGACTAACGTTGTTTCGCATGCAGATTTTCCGGTCGATTGACTCCA	360
1	Q I F R S I D S	8
361	ACTCTGTAAAGGTTTCCCCAAAGATCCCAAAGAAGCCACTAACAAGGCAAGATATTGC	420
8	N S V K G F P K D P K E A T N K	24
421	TTCTAAATGTTTCTTCTATAAAACGCAAGCCATCGATATGCTGTAAGCTAACCATTGTGT	480
	Intron 2	
481	TGACCATTTCTTGTAACAGAATCTAGTTTGTGGCAAGAATGTGCTGATAGATATGAGCAT	540
1	N L V C G K N V L I D M S I	9
541	ACATACTGCCTATGTAAAGGCAATCCGAGCTGCCCAACATTTTCATCTACATTGAGAACCA	600
9	H T A Y V K A I R A A Q H F I Y I E N Q	29
601	GTA CTTCTAGGGTCTCATAACAATTGGAATACTACCAAGATTTAGGTGAGGATTCTAA	660
29	Y F L G S S Y N W N N Y Q D L G	45
661	AAATACTGATTTAAGGGGAAACAAATTTCTTTTCTTTAATGAACCTCTGAATCAATCTG	720
721	TCTGGAATACTTTCAACATAATTCAGTCTGATGTCACAACTGACTAGAAAGTCCATGTGA	780
781	TAAAAGATTTCGATACTTGCTTCATATCTTGTTTAGTTTTCTTCTCTTTGTTATATTT	840
	Intron 3	
841	GAGCTGCTGAAGTCAAGCCCACTTCTTGATGGCGATTGTGAATTCAAAAGTCTTCTTTT	900
901	ATTGATCACCATGACATGTTTGAAGTAAGAATTACTATATAATGATTGTGTAATCTGC	960
961	TTTCTATCTCTAGGTGCAAATAACTTGATACCGATGGAGATTGCTCTAAAAATTGCCAAC	1020
1	G A N N L I P M E I A L K I A N	16
1021	AAAATACGGGCAAATGAGAGGTTTTCAGTATATATAGTTGTTCCCTATGTGGCCAGAGGGT	1080
16	K I R A N E R F S V Y I V V P M W P E G	36
1081	GTTCCAACCACTACTGC	1097
36	V P T S T	41

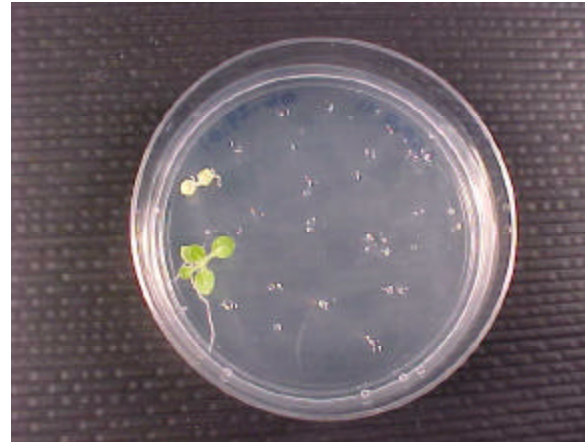
Figure 10. Confirming the incorporation of the transgene into the tobacco genome by PCR analysis. (A) A PCR strategy made it possible to distinguish the transgene from the endogenous gene. Primers designed to amplify a 583 bp fragment of the partial PLD cDNA (AF195614) transgene amplified a 1097 bp segment of the endogenous gene. The genomic region encompassed three introns (I1, I2, and I3) and four exons (E1, E2, E3 and E4). (B) Analysis of PCR products amplified from genomic DNA of transgenic T₀ plants by electrophoresis in a 2% agarose gel and stained with ethidium bromide. M=DNA Markers; wt=PCR product using DNA extracted from wild type T. xanthi leaves as template; 1-13=PCR products using DNA extracted from ASPLD β 1 T₀ T. xanthi leaves as template (antisense PLD or ASPLD1-13); '-'= no template control; '+'=Partial PLD clone (AF195614) amplified to mark the position of the partial PLD cDNA. (C) The 1097 bp nucleotide segment and amino acid sequence of the endogenous gene (Accession No. AY138862). This region contains three introns. The predicted amino acid sequence was generated using DNASIS v2.1. The underlined residues indicate possible alternate splicing sites, although splice acceptor consensus (AG) and splice donor consensus (GT) predict that intron 1 begins at position 262 and that intron 3 begins at position 647.

Segregation Study of the T₁ Progeny

Seeds collected from the T₀ generation of ASPLD7, 9, 11 and 13 plants were germinated on media with or without kanamycin. The results are shown in Table 1. Germinating the seeds without selection revealed that seed viability needed to be taken into account. The transgenic lines ASPLD7 and 13 have very high germination percentages: 100% of the seedlings germinated. However, seeds from ASPLD9 and 11 had 12% and 6% respectively of seeds that were not able to germinate. The percentages shown for seeds germinated with 100 µg/ml kanamycin were therefore adjusted to take into account seed viability (as shown when no selection is used). Wild type and TR1 seed were germinated as controls alongside the transgenic seed in this study. Although controls were sometimes able to germinate when grown on 100 µg/ml kanamycin, none of them survived beyond day 14. Presumably the wild type seeds relied on storage compounds to germinate but the resulting seedlings were unable to sustain themselves on the kanamycin-containing media and died shortly after germination. The percentages determined when seeds were germinated on kanamycin were used to establish ratios in an attempt to understand how the transgene was segregating. The antisense transgene appeared to be segregating following a Mendelian pattern in transgenic tobacco plant lines ASPLD7 and ASPLD11 where the percentages tended towards a 3:1 ratio (Table 1). However, in the case of the ASPLD7 transgenic plants, it would appear that individuals that are homozygous and those that are heterozygous for the transgene germinate on



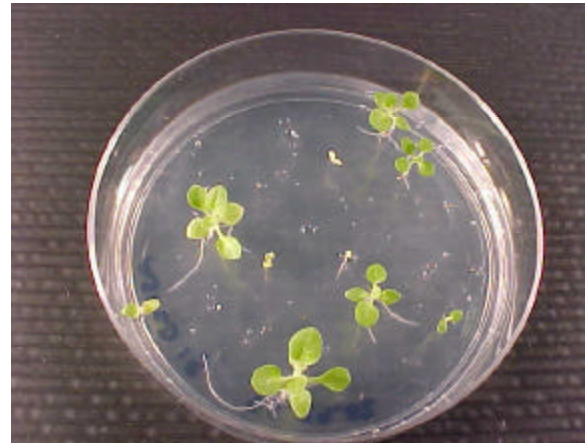
ASPLD7



ASPLD11



ASPLD9



ASPLD13

Figure 11. Transgenic ASPLD7, 9, 11, and 13 seeds (T1 generation) germinated on plates with 100 $\mu\text{g/mL}$ kanamycin to investigate segregation of the transgene.

Table 1. Numbers of germinated ASPLD 7, 9, 11 and 13 T1 seedlings. Seeds were germinated on plates with or without kanamycin and seed germination was observed up until 14 days after planting. The ratios of kanamycin resistant to kanamycin sensitive seedlings were as follows: 3:1 (ASPLD 7) \approx 1 (ASPLD 9), 1:3(ASPLD 11) and 1:1 (ASPLD 13). Seed viability was taken into account.

	ASPLD7	ASPLD9	ASPLD11	ASPLD13
100 µg/ml Kanamycin	74	84	19	45
No selection	100	88	94	100

kanamycin. Hence 74% germinate (Figure 11). Only homozygotes for the transgene germinate in the case of the transgenic line ASPLD11 (20%). Presumably, the heterozygotes do not survive in this case. One copy of the transgene may not sufficient for the progeny to germinate on 100 µg/ml kanamycin. It is therefore possible that the ASPLD7 line may have 2 copies that are located very close together so as to cause them to segregate as one, but in so doing, enabling the heterozygous population to survive on kanamycin. ASPLD9 was found to germinate at 95% on kanamycin. It is possible that this particular line contains multiple copies of the transgene, making it an unlikely event for the transgene to not be passed on to the progeny. Therefore very few seeds do not contain at least one copy of the transgene. As for ASPLD13, half on the progeny were

able to germinate on kanamycin (Figure 11). It is therefore likely that the homozygotes for the transgene are able to germinate but that only half the heterozygotes are able to do so. Maybe this is also the result of multiple transgene copies located close to each other within the genome and possibly only half of the heterozygous are able to germinate due to some kind of dosage effect if both copies are not functionally expressed. Furthermore, segregation sometimes does not stabilize until as late as the T₄ generation (Dr Feldman, pers.comm.). It is therefore likely that these numbers will become more interpretable as further generations are grown.

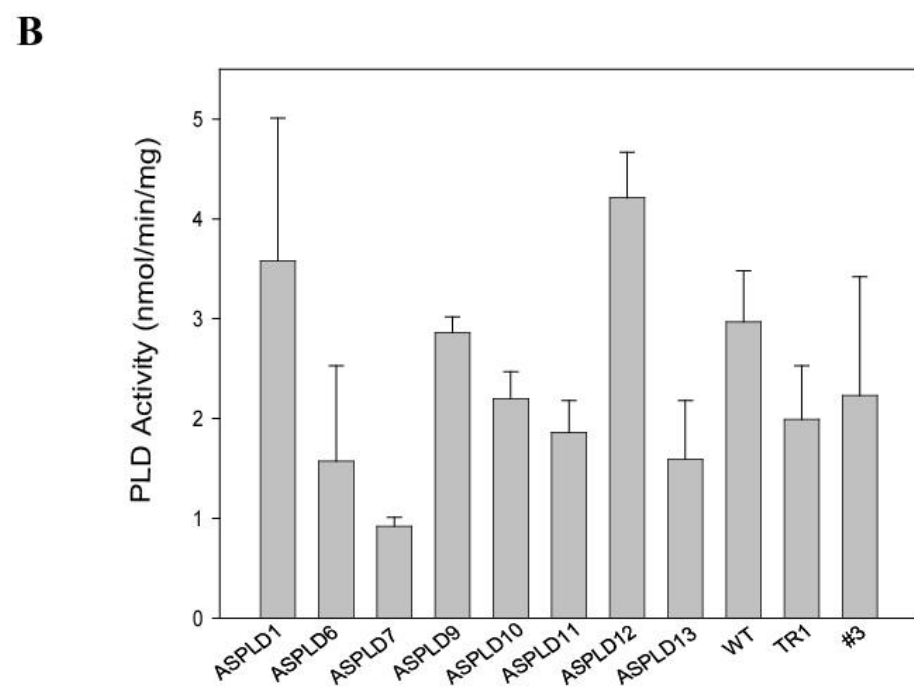
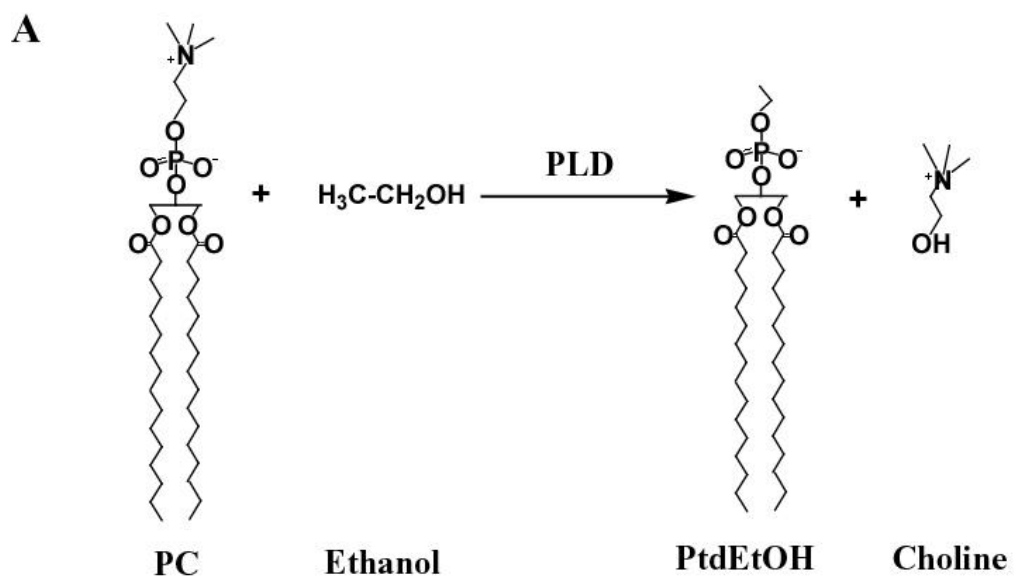
PLD Activity Screen of the Antisense PLD Plants

Crude leaf homogenates of the fully grown transgenic plants were used to survey the plants for differences in PLD activity compared with the PLD activity in wild type controls, controls that had been subjected to the regeneration process (TR1) and transgenic plants that had shown negative results in the PCR screening for the insertion of the transgene (eg. ASPLD3). Assays were performed in micromolar calcium conditions, in the presence of PIP₂/PE vesicles at pH 7.0. These conditions select specifically for PLD β /? activity (Qin *et al.*, 1997). PLD activity was measured either as transphosphatidylation (Figure 12A) or hydrolysis of NAPE (Figure 12C). In the transphosphatidylation reaction radiolabelled PC was used as a substrate. Alcohol was supplied in the form as ethanol. The enzyme catalyzed the formation of phosphatidylethanol and released free choline. Quantification of radiolabelled phosphatidylethanol by radiometric scanning was used to measure PLD activity. For the hydrolysis reaction radiolabelled NAPE was used as a substrate and the enzyme catalyzed

the hydrolysis of NAPE thus forming phosphatidic acid and NAEs. Quantification of radiolabelled NAEs by radiometric scanning was used to measure the hydrolytic activity of PLD.

In the case of the transphosphatidylation experiments, no significant difference in PLD activity was observed. PLD activity appeared to be similar in the transgenics and the controls with the exception of sample ASPLD7 where activity was lower (Figure 12B) although not significant ($P>0.05$). Activity was also somewhat reduced in the ASPLD6, 11 and 13 samples although less notably so. However, PLD activity was not completely lost in any of the antisensed plants. In the case of the ASPLD1, 8 and 12 samples, PLD activity even appeared elevated when compared to the controls. PLD activity in ASPLD9 and 10 samples was closest to the wild type activity.

However, when screening the transgenic plants using the hydrolytic assay, two plants were found to have significantly reduced activity when compared to the controls. NAPE hydrolysis activity was significantly reduced in ASPLD1 and ASPLD9. Activity was also reduced in ASPLD7 although not significantly so ($P>0.05$). The other transgenic plants showed no difference in their ability to hydrolyze NAPE *in vitro*.



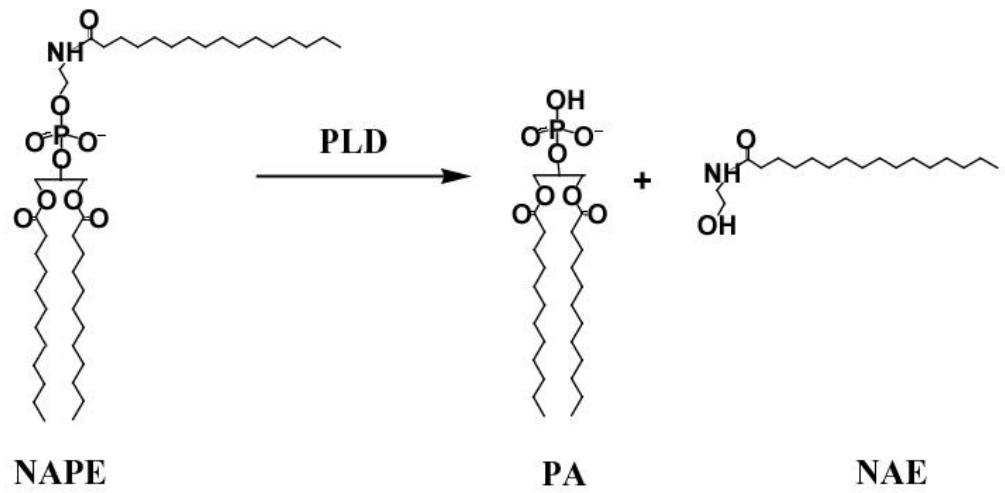
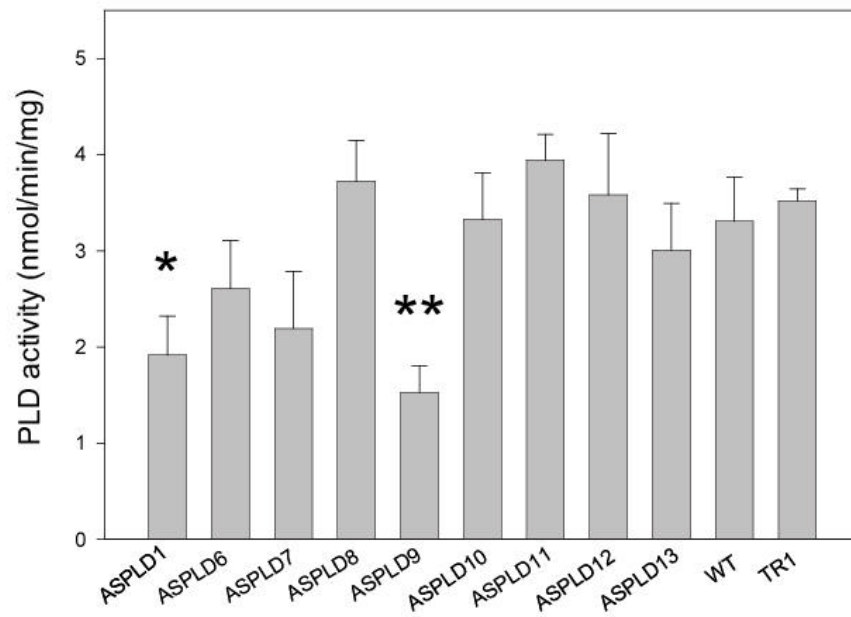
C**D**

Figure 12. The trademark of most PLDs is their ability to perform transphosphatidylation of a primary alcohol. In this scenario (A) the phosphatidic group of phosphatidylcholine is transferred to ethanol by PLD to form phosphatidylethanol (PtdEtOH) and free choline. Alternatively, PLD isoforms β and γ are also able to catalyze the hydrolysis of phospholipids such as NAPE, generating phosphatidic acid (PA) and in this case NAEs (C). Both assays were used to measure PLD activity in the transgenic plants. (B) PLD activity in wild type and ASPLD tobacco extracts. Activity was measured as transphosphatidylation of L-alpha-dipalmitoyl, [dipalmitoyl-1- ^{14}C] phosphatidylcholine and was quantified as production of radiolabelled phosphatidylethanol. Activity was measured in crude homogenates of transgenic T_0 plants (ASPLD1, 6, 7, 9-13) and compared to activity in crude homogenates of wild type tobacco (WT), regeneration controls (TR1) and non-transgenic plants (#3, that tested negative during the PCR screen to confirm insertion of the transgene). (D) PLD activity in wild type and ASPLD tobacco extracts. Activity was measured as hydrolysis of 1,2-dilauryl-sn-glycero-3-phospho(N-[2- ^{14}C]palmitoyl) ethanolamine and was quantified as production of radiolabelled NAEs. Activity was measured in crude homogenates of transgenic T_0 plants (ASPLD1, 6, 7, 9-13) and compared to activity in crude homogenates of wild type tobacco (WT), regeneration controls (TR1) and non-transgenic plants (#3, that tested negative during the PCR screen to confirm insertion of the transgene). In both cases assays were conducted under conditions optimal for PLD β/γ activity (Qin et al., 1997) and data are means and \pm SD of triplicate measurements. ** indicates $p < 0.005$; * indicates $p < 0.05$.

Root Growth Rate

Seeds collected from the T₀ generation of the ASPLD7, 9, 11 and 13 plants were germinated on plant media plates alongside wild type controls and root length was measured at intervals until day 11 after plating. The results show that the transgenic seeds germinated after the wild type seeds. These seeds were germinated on plates without kanamycin as the antibiotic is known to interfere with root development (Dr. Tripathy, pers. comm.). Therefore the results also include data from progeny that do not carry the transgene. Kanamycin was applied after day 11 to select for seedlings carrying the transgene. However, this screening method proved unpractical as all seedlings showed signs of difficulty growing in the presence of the kanamycin. Presumably, seedlings need to degrade the kanamycin progressively as they grow and are unable to degrade the antibiotic effectively when placed in it at a later time. The results therefore include data from seedlings that undoubtedly did not carry the transgene. One could therefore project that the differences would be even more marked if these data could be removed especially in plant lines ASPLD 11 and 13 where so many of the progeny do not germinate on kanamycin in the previous germination studies. The growth rates were deduced by regression analysis and are as follows: 2.41 mm/day for wild type tobacco, 1.90 mm/day for ASPLD7, 2.18 mm/day for ASPLD9, 2.85 mm/day for ASPLD11 and 2.35 mm/day for ASPLD13. The root growth rate in seedlings from ASPLD7, 9, 11 and 13 is similar to that of the wild type seedlings although ASPLD7 and 9 have somewhat lower root growth rates and ASPLD11 and 13 have somewhat higher root growth rates. Since 80% of the seedlings in plant line ASPLD11 do not germinate on kanamycin. It is

also highly likely that the germination data is skewed by the high number of seedlings that do not carry the transgene.

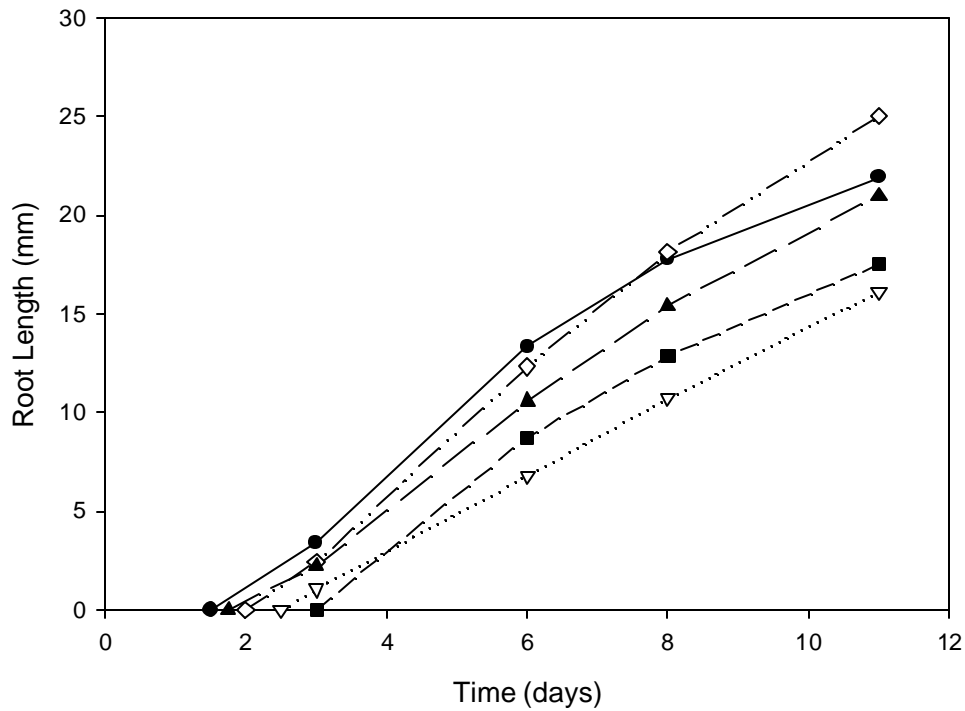


Figure 13. Root growth rate of wt and ASPLD tobacco seedlings. Fifty seedlings were germinated per sample. (●) wild type tobacco, (▽) ASPLD7, (◊) ASPLD9, (▲) ASPLD11, (◻) ASPLD13. Standard deviation was never above 5.25 mm at each timepoint. Statistical analysis revealed that on day 3 after plating the root lengths of the different samples were significant at $P < 0.005$; on day 6 and 8 after plating the root lengths of the different samples were significant at $P < 0.005$ with the exception of ASPLD11 that was not significantly different from the wt; on day 11 after plating the root lengths of the different samples were significant at $P < 0.005$ with the exception of ASPLD13 that was not significantly different from the wt. The growth rates were deduced by regression analysis and are as follows: 2.41 mm/day for wild type tobacco, 1.9 mm/day for ASPLD7, 2.18 mm/day for ASPLD9, 2.85 mm/day for ASPLD11 and 2.35 mm/day for ASPLD13.

CHAPTER 4

DISCUSSION

Based on overall similarity, Nt10B1 is most likely a partial PLD β 1. It would appear to be missing the C2 calcium-binding domain at the amino terminus of the protein. Unlike the other domains, the C2 domain is not fully conserved among all PLDs. The number of calcium-binding acidic residues varies. In *Arabidopsis*, PLD β , γ and δ share the same number of acidic residues within the C2 calcium-binding domain, whereas PLD α lacks one or more and PLD α 4 in particular has none at all (Qin and Wang, 2002). Furthermore, it has been suggested that this is possibly how different PLDs are active at different calcium concentrations (Qin and Wang, 2002). For instance, PLD β and γ not only share the same number of calcium-binding residues, they also share the same micromolar calcium concentration for optimal activity. PLD δ , however, is a calcium-independent enzyme (Qin and Wang, 2002) whereas it also shares the same number of acidic residues within this region, suggesting that in this enzyme's case, this region could be redundant. The PLD assays, conducted using recombinant protein from the "Expres" clones, suggest that the calcium-binding domain is indeed necessary for PLD β / γ enzyme activity. The calcium-binding domain or C2 domain can also bind phospholipids, polyphosphoinositides and proteins (Zheng *et al.*, 2000). It is therefore possible that it is necessary for binding one of these other factors, if not calcium itself and that without co-factor binding, PLD β / γ activity is lost.

Transgenic tobacco plants were developed with the partial tobacco PLD cDNA clone in antisense orientation to investigate the role of PLD in plants. The intention was to generate plants that would lack PLD activity and would consequently display an altered phenotype. The partial PLD clone shares most identity with PLD β 1 from tomato (91%), but has identity with both *Arabidopsis* PLD isoforms β (72%) and γ (68%). The ASPLD plants generated in this study do not outwardly show a particular phenotype. They are indistinguishable in phenotype from the wild type tobacco (cv Xanthi). Although PLD transphosphatidylation activity of crude leaf homogenates from the transgenics did not vary significantly from wild type levels and NAPE hydrolysis activity was significantly reduced in two of the transgenics, no plants were found where PLD activity was completely knocked out. The assay conditions used in this study (micromolar calcium, presence of PIP₂ and PE, pH 7.0) were specific for the PLD isoforms β and γ (Pappan, *et al.*, 1998). Both β and γ isoforms require the same conditions for optimal activity. Both isoforms use the same substrates, although they have slightly different substrate preferences (Pappan, *et al.*, 1998). These factors make it impossible to attribute activity to one isoform over the other. At the molecular level PLD β and γ are also closely related. In *Arabidopsis* PLD β shares 70% identity with PLD γ 1 and 69% identity with PLD γ 2. No PLD γ isoforms have yet been found in either tomato or tobacco. However, tomato PLD β 1 and β 2 share 71% identity, which makes them as similar to each other as the *Arabidopsis* PLD β and γ isoforms.

Table 2. *Arabidopsis thaliana* PLD β 1 similarity at the DNA level with other *Arabidopsis* PLD isoforms over the region that shares identity with the partial tobacco PLD clones (AF195614) used to generate the antisense (ASPLD) plants. In brackets the length of the homologous region is given. PLD β 2 (Protein Accession AAF02803) from *Arabidopsis* was omitted as the cDNA has yet to be cloned.

	Identity with PLD β 1 (U84568) over the region that shares identity with tobacco partial PLD clone AF195614 (length of shared region shown in brackets)	Identity with full length PLD β 1
PLD γ 1 (AF027408)	76% (203/267 bp)	76% (388/507 bp)
PLD γ 2 (AF138281)	75% (226/299 bp)	76% (387/508 bp)
PLD δ a (AB031047)	78% (66/84 bp)	76% (145/189 bp)
PLD δ (AF322228)	78% (64/84 bp)	76% (145/189 bp)
PLD ζ (AF411833)	91% (31/34 bp)	91% (31/34 bp)
PLD α (U36381)	No identity	No identity

These findings raise the question as to whether these two isoforms should be classed together or whether they may in fact belong to the same class. Table 2 shows the relation between PLD isoforms within *Arabidopsis*, more specifically between the regions of these PLDs that align with the fragment used to antisense the ASPLD plants. In tomato, PLD β 1 shares 71% identity with PLD β 2 over this region. In *Arabidopsis*, PLD β 1 shares 76% and 75% identity with PLD γ 1 and γ 2 respectively over this region. It is therefore possible that by using the partial PLD clone (which shares 91% identity with tomato PLD β 1) to antisense the tobacco plants, that only PLD β 1 was in fact antisensed. This would explain why the PLD β / γ -type activity was not knocked out in these transgenics. The plant was able to rely on one of the other members of the PLD β (and/or other PLD γ) family to maintain PLD activity.

Total leaf homogenates were assayed for functional PLD activity in conditions optimal for PLD β / γ -type activity using two different reactions. Firstly, the activity was measured as transphosphatidylation of PC using ethanol as the primary alcohol supplied. In this case, no significant decrease in PLD β / γ -type activity was observed. However, when measured as NAPE hydrolysis activity was significantly reduced in ASPLD1 and 9. These preliminary results raise an interesting question. They suggest that the transphosphatidylation activity and NAPE hydrolysis activity are not both affected by the presence of the antisense fragment. Furthermore, expression data of PAL2 (the defense gene encoding phenylalanine-ammonia lyase) from the ASPLD and wild type tobacco plants, when treated either the fungal elicitor xylanase or water as a control, reveals additional information (Tripathy, unpublished) (see Appendix). In this study PAL2

expression was found to be significantly down-regulated in ASPLD9 (around 80% reduction), 11 (50%), 12 (35%) and 13 (50%) when elicited with xylanase. Meanwhile, PAL2 expression was not affected in ASPLD3 (one of the ASPLD plants that tested negative when PCR screened for insertion of the transgene) or in the regeneration control TR1. Interestingly, background expression of PAL2 in ASPLD13 was 4-5 fold higher than in wild type, whereas PAL2 expression in ASPLD 9, 11, 12 and 13 when treated with water was at wild type level. These results suggest that the ASPLD9, 11, 12, and 13 plants are effectively PLD-antisensed since signal transduction between the elicitor xylanase and PAL2 expression is not as effective as in the wild type and control plants. As mentioned earlier, *in vitro* NAPE hydrolysis activity was reduced in ASPLD9. It is therefore possible that basal PLD activity is maintained in the ASPLD plants by isoforms closely related to PLD β 1. The expression of these isoforms is most likely not affected by the antisense partial PLD fragment. However, upon elicitation with xylanase, a rapid turnover must ensue to generate the downstream signal for PAL2 expression. At this point, the overlapping isoforms can no longer compensate and PAL2 expression is reduced as a consequence. Alternatively, all the β/γ isoforms may contribute towards the transphosphatidylation activity observed *in vitro* but it may be that the PLD β 1 alone is responsible for NAPE hydrolysis and generating NAEs as previously reported (Tripathy *et al.*, 1999) in the downstream signaling events in an elicitor-induced response. The latter hypothesis is plausible since it has already been shown in tomato that PLD β 1 rapidly and specifically accumulates in response to treatment with xylanase (Laxalt *et al.*, 2001). In this study, they were able to show that PLD β 1 mRNA increased up to 2 h after

treatment with xylanase, before decreasing back to background levels over 72 h.

Together, these results suggest that the function of PLD β 1 is most likely signal transduction. Future studies plan to include the synthesis of a probe specific to the partial tobacco PLD fragment in order to investigate expression levels of the isoform affected in the ASPLD plants. Furthermore, performing the same functional assays as described above on total leaf extracts of T₁, T₂ and T₃ plants will confirm this difference in the effect of the transgene on transphosphatidylolation activity versus NAPE hydrolysis.

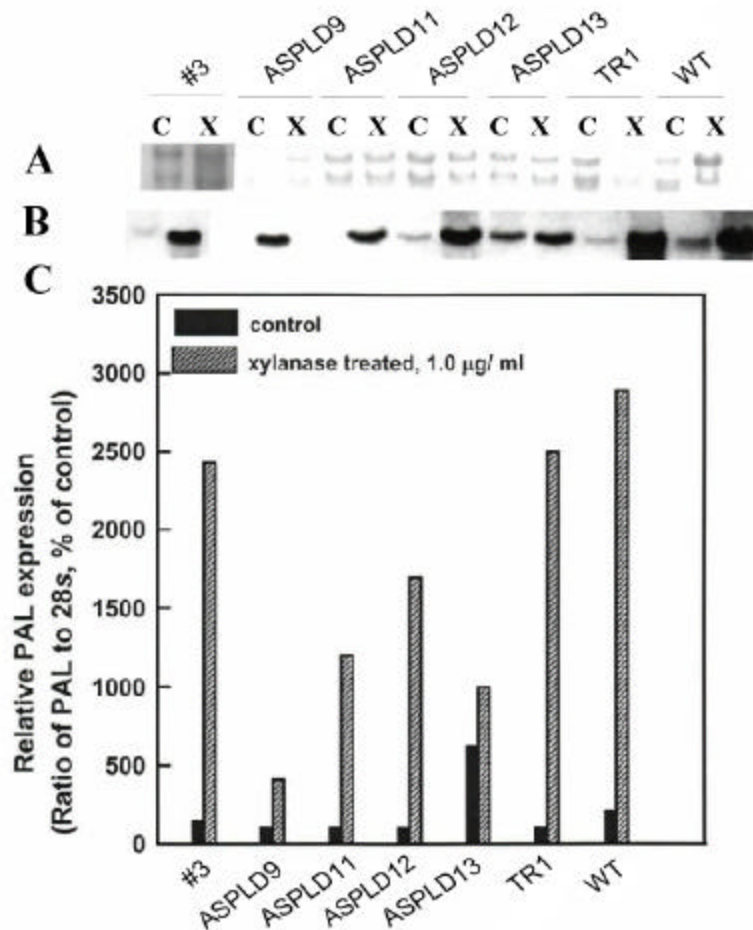
The germination and root growth study revealed that ASPLD transgenics germinate later than wild type tobacco seeds. PLD has been shown to be associated with the cytoskeletal elements of the cell and furthermore, has been implicated in intracellular trafficking (Gardiner *et al.*, 2001; Munnik and Musgrave, 2001). It is therefore possible that the observed delay in germination could be due to reduced PLD activity resulting in slower trafficking and possibly slower membrane deposition. PLD has also been implicated in mitosis (Rose *et al.*, 1995; Gardiner *et al.*, 2001). It is therefore possible that cell division could be affected. However, in both of these scenarios, one would expect a decreased growth rate throughout plant development, which was not generally noted in the ASPLD transgenic tobacco plants. It may be that PLD β 1 is more specifically involved in seed germination. Studies in castor bean revealed that PLD α protein expression levels increase shortly after imbibition (Wang *et al.*, 1993). Not only did PLD α expression levels increase, three other PLDs active in a conditions were shown to be expressed in a growth-stage-dependant manner throughout germination and seed development (Dyer *et al.*, 1994). However, at the time of this particular investigation the

PIP₂-dependant isoforms PLD β and γ were unknown. PIP₂-dependant PLD activity was shown to increase from 2 to 5 days after germination in microsomal fractions of hypocotyls in *Brassica napus* seedlings (Novotna *et al.*, 2000). These results suggest that at least one of the β/γ isoforms is active during germination although no β/γ -type activity was found in the seeds whereas PLD α was recorded at this time in the same study. Further studies need to be done to address this question in tobacco. It would be interesting to study the localization of these different isoforms throughout seed germination and development to identify where they may be active. Wang *et al.* were able to study PLD isoform localization within leaves using specific antibodies and fluorescence detection (Sang *et al.*, 2001). It would be interesting to apply this study to germinating seeds to find where the different isoforms are localized during germination. At this time, further research needs to be done in order to determine whether this delay in germination comes as a result of down-regulated intracellular trafficking, degradation of oil body phospholipids, cell division or simply as a result of down-regulated cell signaling.

In summary, this study has isolated a cDNA clone Nt10B1 with 91% identity at the amino acid level with PLD β 1 from tomato but lacking the *N* terminal calcium-binding domain. Transgenic plants developed with the partial tobacco PLD fragment used to screen the library are therefore most likely PLD β 1-antisensed based on sequence identity. When total leaf extracts were used to measure PLD activity in β/γ -type conditions as transphosphatidylation or NAPE hydrolysis, there was no significant difference in transphosphatidylation whereas NAPE hydrolysis activity was significantly reduced in

ASPLD1 and 9. Furthermore, based on previous work on the expression of the PLD β 1 isoform in tomato and PAL2 expression data, it would appear that PLD β 1 activity in the ASPLD plants might be down-regulated, although this could not be confirmed *in vitro* as other members of the PLD β /? family share the same enzymatic requirements. Down-regulating PLD β 1 seems to have an affect during germination. It is unclear what role PLD β 1 plays during seed germination. However, the enzyme might also be involved in signal transduction in the seed. Expression studies of the PLD β 1 isoform and its close relatives, together with elicitor treatment studies should confirm its function in signal transduction and its down-regulation in the ASPLD transgenics.

APPENDIX



Appendix 1 Analysis of xylanase-induced PAL2 mRNA transcript abundance in ASPLD β 1 transgenics (ASPLD9, 11-13) and transformation (TR1, #3 tested PCR negative) and wild type controls. Total RNA isolation (8 h post treatment on leaves), northern blot preparation and RNA quantification were carried out as reported earlier (Tripathy *et al.*, 1999). Fully expanded alternate tobacco leaves were infiltrated either with xylanase (X, 1.0 $\mu\text{g}/\text{mL}$) or water as control (C). (A) Methylene blue-stained blot showing relative amount of total RNA loaded. (B) Northern blot probed with tobacco PAL2. (C) Quantitative representation of relative PAL2 mRNA transcript abundance (normalized to 28S rRNA and percent of control levels of PAL2).

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